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<p>(21) International Application Number: PCT/US99/14311</p> <p>(22) International Filing Date: 22 June 1999 (22.06.99)</p> <p>(30) Priority Data: 60/091,403 29 June 1998 (29.06.98) US</p> <p>(71) Applicant (for all designated States except US): U.S. MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES [US/US]; Dept. of the Army, 504 Scott Street, Frederick, MD 21702 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): HART, Mary, Katherine [US/US]; 2517 Waterside Drive, Frederick, MD 21701 (US). WILSON, Julie, A. [US/US]; 2419 Lakeside Drive, Frederick, MD 21702 (US). PUSHKO, Peter [US/US]; 917 Seminole Road, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US]; 6936 Eylers Valley Flint Road, Sabillasville, MD (US). SCHMALJOHN, Alan, L. [US/US]; 7613 Irongate Drive, Frederick, MD (US).</p> <p>(74) Agents: HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 (US) et al.</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>
<p>(54) Title: EBOLA VIRION PROTEINS EXPRESSED FROM VENEZUELAN EQUINE ENCEPHALITIS (VEE) VIRUS REPLICONS</p>		
<p>(57) Abstract</p> <p>Using the Ebola GP, NP, VP24, VP30, VP35 and VP40 virion proteins, a method and composition for use in inducing an immune response which is protective against infection with Ebola virus is described.</p>		

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TITLE OF THE INVENTION

Ebola Virion Proteins Expressed from Venezuelan Equine
Encephalitis (VEE) Virus Replicons

INTRODUCTION

Ebola viruses, members of the family Filoviridae, are associated with outbreaks of highly lethal hemorrhagic fever in humans and nonhuman primates. The natural reservoir of the virus is unknown and there currently are no available vaccines or effective therapeutic treatments for filovirus infections. The genome of Ebola virus consists of a single strand of negative sense RNA that is approximately 19 kb in length. This RNA contains seven sequentially arranged genes that produce 8 mRNAs upon infection (Fig. 1). Ebola virions, like virions of other filoviruses, contain seven proteins: a surface glycoprotein (GP), a nucleoprotein (NP), four virion structural proteins (VP40, VP35, VP30, and VP24), and an RNA-dependent RNA polymerase (L) (Feldmann et al. (1992) *Virus Res.* **24**, 1-19; Sanchez et al., (1993) *Virus Res.* **29**, 215-240; reviewed in Peters et al. (1996) *In Fields Virology*, Third ed. pp. 1161-1176. Fields, B. N., Knipe, D. M., Howley, P.M., et al. eds. Lippincott-Raven Publishers, Philadelphia). The glycoprotein of Ebola virus is unusual in that it is encoded in two open reading frames. Transcriptional editing is needed to express the transmembrane form that is incorporated into the virion (Sanchez et al. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 3602-3607;

1 Volchkov et al, (1995) *Virology* **214**, 421-430. The
2 unedited form produces a nonstructural secreted
3 glycoprotein (sGP) that is synthesized in large
4 amounts early during the course of infection. Little
5 is known about the biological functions of these
6 proteins and it is not known which antigens
7 significantly contribute to protection and should
8 therefore be used to induce an immune response.

9 Recent studies using rodent models to evaluate
10 subunit vaccines for Ebola virus infection using
11 recombinant vaccinia virus encoding Ebola virus GP
12 (Gilligan et al., (1997) *In Vaccines* **97**, pp. 87-92.
13 Cold Spring Harbor Laboratory Press, Cold Spring
14 Harbor, N.Y.), or naked DNA constructs expressing
15 either GP or sGP (Xu et al. (1998) *Nature Med.* **4**, 37-
16 42) have demonstrated the protective efficacy of Ebola
17 virus GP in guinea pigs. (All documents cited herein
18 *supra* and *infra* are hereby incorporated in their
19 entirety by reference thereto.) Additionally, Ebola
20 virus NP and GP genes expressed from naked DNA
21 vaccines (Vanderzanden et al., (1998) *Virology* **246**,
22 134-144) have elicited protective immunity in BALB/c
23 mice. However, successful vaccination of nonhuman
24 primates with individual Ebola virus genes has not
25 been demonstrated. Therefore, there exists a need for
26 a vaccine which is efficacious for protection from
27 Ebola virus infection.

28

29 SUMMARY OF THE INVENTION

30 The present invention satisfies the need
31 discussed above. The present invention relates to a
32 method and composition for use in inducing an immune
33 response which is protective against infection with
34 Ebola virus.

35 Because the biological functions of the
36 individual Ebola virus proteins are not known and the
37 immune mechanisms necessary for preventing and

1 clearing Ebola virus infection are not well
2 understood, it was not clear which antigens
3 significantly contribute to protection and should
4 therefore be included in an eventual vaccine candidate
5 to induce a protective immune response. We evaluated
6 the ability of packaged Venezuelan equine encephalitis
7 (VEE) virus replicons expressing GP, NP, VP40, VP35,
8 VP30 and VP24 virion proteins of Ebola virus to elicit
9 protective immunity in two strains of mice which
10 differ at the major histocompatibility locus. There
11 are no published reports of the VP proteins having
12 been assayed as antigens for the production of an
13 immune response in a mammal.

14 The VEE virus replicon (Vrep) is a genetically
15 reorganized version of the VEE virus genome in which
16 the structural protein genes are replaced with a gene
17 from an immunogen of interest, such as the Ebola virus
18 virion proteins. This replicon can be transcribed to
19 produce a self-replicating RNA that can be packaged
20 into infectious particles using defective helper RNAs
21 that encode the glycoprotein and capsid proteins of
22 the VEE virus. Since the packaged replicons do not
23 encode the structural proteins, they are incapable of
24 spreading to new cells and therefore undergo a single
25 abortive round of replication in which large amounts
26 of the inserted immunogen are made in the infected
27 cells. The VEE virus replicon system is described in
28 U.S. Patent to Johnston *et al.*, patent no. 5,792,462
29 issued on August 11, 1998.

30 For our purposes, each of the Ebola virus genes
31 were individually inserted into a VEE virus replicon
32 vector. The VP24, VP30, VP35, and VP40 genes of Ebola
33 Zaire 1976 (Mayinga isolate) were cloned by reverse
34 transcription of RNA from Ebola-infected Vero E6 cells
35 and viral cDNAs were amplified using the polymerase
36 chain reaction. The Ebola Zaire 1976 (Mayinga isolate)
37 GP and NP genes were obtained from plasmids already
38 containing these genes (Sanchez, A. *et al.*, (1989)

1 *Virology* **170**, 81-91; Sanchez, A. et al., (1993) *Virus*
2 *Res.* **29**, 215-240) and were subcloned into the VEE
3 replicon vector.

4 After characterization of the Ebola gene
5 products expressed from the VEE replicon constructs in
6 cell culture, these constructs were packaged into
7 infectious VEE virus replicon particles (VRPs) and
8 subcutaneously injected into BALB/c and C57BL/6 mice.
9 As controls in these experiments, mice were also
10 immunized with a VEE replicon expressing Lassa
11 nucleoprotein (NP) as an irrelevant control antigen,
12 or injected with PBS buffer alone. The results of this
13 study demonstrate that VRPs expressing the Ebola GP,
14 NP, VP24, VP30, VP35 or VP40 genes induced protection
15 in mice and may provide protection in humans.

16

17 Therefore, it is one object of the present
18 invention to provide a DNA fragment encoding each of
19 the Ebola Zaire 1976 GP, NP, VP24, VP30, VP35, and
20 VP40 virion proteins (SEQUENCE ID NOS. 1-7).

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22 It is another object of the present invention to
23 provide the DNA fragments of Ebola virion proteins in
24 a recombinant vector. When the vector is an
25 expression vector, the Ebola virion proteins GP, NP,
26 VP24, VP30, VP35, and VP40 are produced.

27

28 It is yet another object of the present
29 invention to provide a VEE virus replicon vector
30 comprising a VEE virus replicon and a DNA fragment
31 encoding any of the Ebola Zaire 1976 (Mayinga isolate)
32 GP, NP, VP24, VP30, VP35, or VP40 proteins. The
33 construct can be used as a nucleic acid vaccine or for
34 the production of self replicating RNA.

35

36 It is another object of the present invention to
37 provide a self replicating RNA comprising the VEE
38 virus replicon and any of the Ebola Zaire 1976

1 (Mayinga isolate) RNAs encoding the GP, NP, VP24,
2 VP30, VP35, and VP40 proteins described above. The
3 RNA can be used as a vaccine for protection from Ebola
4 infection. When the RNA is packaged, a VEE virus
5 replicon particle is produced.

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7 It is another object of the present invention to-
8 provide infectious VEE virus replicon particles
9 produced from the VEE virus replicon RNAs described
10 above.

11

12 It is further an object of the invention to
13 provide an immunological composition for the
14 protection of subjects against Ebola virus infection,
15 comprising VEE virus replicon particles containing the
16 Ebola virus GP, NP, VP24, VP30, VP35, or VP40
17 proteins, or any combination of different VEE virus
18 replicons each containing one or more different Ebola
19 proteins selected from GP, NP, VP24, VP30, VP35 and
20 VP40.

21

22 BRIEF DESCRIPTION OF THE DRAWINGS

23 These and other features, aspects, and
24 advantages of the present invention will become better
25 understood with reference to the following description
26 and appended claims, and accompanying drawings where:

27 Figure 1 is a schematic description of the
28 organization of the Ebola virus genome.

29 Figures 2A, 2B and 2C are schematic
30 representations of the VEE replicon constructs
31 containing Ebola genes.

32 Figure 3 shows the generation of VEE viral-like
33 particles containing Ebola genes.

34 Figure 4 is an immunoprecipitation of Ebola
35 proteins produced from replicon constructs.

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DETAILED DESCRIPTION

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In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

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Filoviruses. The filoviruses (e.g. Ebola Zaire 1976) cause acute hemorrhagic fever characterized by high mortality. Humans can contract filoviruses by infection in endemic regions, by contact with imported primates, and by performing scientific research with the virus. However, there currently are no available vaccines or effective therapeutic treatments for filovirus infection. The virions of filoviruses contain seven proteins: a membrane-anchored glycoprotein (GP), a nucleoprotein (NP), an RNA-dependent RNA polymerase (L), and four virion structural proteins (VP24, VP30, VP35, and VP40). Little is known about the biological functions of these proteins and it is not known which antigens significantly contribute to protection and should therefore be used in an eventual vaccine candidate.

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Replicon. A replicon is equivalent to a full-length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be inserted downstream of the 26S promoter into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be inserted into this cloning site. The RNA that is transcribed from the replicon is capable of replicating and expressing viral proteins in a manner that is similar to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed from

1 the 26S promoter in the replicon. This system does not
2 yield any progeny virus particles because there are no
3 viral structural proteins available to package the RNA
4 into particles.

5 Particles which appear structurally identical to
6 virus particles can be produced by supplying
7 structural protein RNAs *in trans* for packaging of the
8 replicon RNA. This is typically done with two
9 defective helper RNAs which encode the structural
10 proteins. One helper consists of a full length
11 infectious clone from which the nonstructural protein
12 genes and the glycoprotein genes are deleted. This
13 helper retains only the terminal nucleotide sequences,
14 the promoter for subgenomic mRNA transcription and the
15 sequences for the viral nucleocapsid protein. The
16 second helper is identical to the first except that
17 the nucleocapsid gene is deleted and only the
18 glycoprotein genes are retained. The helper RNAs are
19 transcribed *in vitro* and are co-transfected with
20 replicon RNA. Because the replicon RNA retains the
21 sequences for packaging by the nucleocapsid protein,
22 and because the helpers lack these sequences, only the
23 replicon RNA is packaged by the viral structural
24 proteins. The packaged replicon particles are released
25 from the host cell and can then be purified and
26 inoculated into animals. The packaged replicon
27 particles will have a tropism similar to the parent
28 virus. The packaged replicon particles will infect
29 cells and initiate a single round of replication,
30 resulting in the expression of only the virus
31 nonstructural proteins and the product of the
32 heterologous gene that was cloned in the place of the
33 virus structural proteins. In the absence of RNA
34 encoding the virus structural proteins, no progeny
35 virus particles can be produced from the cells
36 infected by packaged replicon particles.

37 The Venezuelan equine encephalitis (VEE) virus
38 replicon is a genetically reorganized version of the

1 VEE virus genome in which the genes encoding the VEE
2 structural proteins are replaced with a heterologous
3 gene of interest. In the present invention, the
4 heterologous genes are the GP, NP, or VP virion
5 proteins from the Ebola virus. The result is a self-
6 replicating RNA that can be packaged into infectious
7 particles using defective helper RNAs that encode the
8 glycoprotein and capsid proteins of the VEE virus. The
9 replicon and its use is further described in U.S.
10 Patent no 5,792,462 issued to Johnston *et al.* on
11 August 11, 1998.

12 **Subject.** Includes both human, animal, e.g.,
13 horse, donkey, pig, mouse, hamster, monkey, chicken,
14 and insect such as mosquito.

15 In one embodiment, the present invention relates
16 to DNA fragments which encode any of the Ebola Zaire
17 1976 (Mayinga isolate) GP, NP, VP24, VP30, VP35, and
18 VP40 proteins. The GP and NP genes of Ebola Zaire were
19 previously sequenced by Sanchez *et al.* (1993, *supra*)
20 and have been deposited in GenBank (accession number
21 L11365). A plasmid encoding the VEE replicon vector
22 containing a unique ClaI site downstream from the 26S
23 promoter was described previously (Davis, N. L. *et*
24 *al.*, (1996) *J. Virol.* **70**, 3781-3787; Pushko, P. *et*
25 *al.* (1997) *Virology* **239**, 389-401). The Ebola GP and
26 NP genes from the Ebola Zaire 1976 virus were derived
27 from PS64- and PGEM3ZF(-)-based plasmids (Sanchez, A.
28 *et al.* (1989) *Virology* **170**, 81-91; Sanchez, A. *et al.*
29 (1993) *Virus Res.* **29**, 215-240). From these plasmids,
30 the BamHI-EcoRI (2.3 kb) and BamHI-KpnI (2.4 kb)
31 fragments containing the NP and GP genes,
32 respectively, were subcloned into a shuttle vector
33 that had been digested with BamHI and EcoRI (Davis *et*
34 *al.* (1996) *supra*; Grieder, F. B. *et al.* (1995)
35 *Virology* **206**, 994-1006). For cloning of the GP gene,
36 overhanging ends produced by KpnI (in the GP fragment)
37 and EcoRI (in the shuttle vector) were made blunt by
38 incubation with T4 DNA polymerase according to methods

1 known in the art. From the shuttle vector, GP or NP
2 genes were subcloned as ClaI-fragments into the ClaI
3 site of the replicon clone, resulting in plasmids
4 encoding the GP or NP genes in place of the VEE
5 structural protein genes downstream from the VEE 26S
6 promoter.

7 The VP genes of Ebola Zaire were previously
8 sequenced by Sanchez et al. (1993, *supra*) and have
9 been deposited in GenBank (accession number L11365).
10 The VP genes of Ebola used in the present invention
11 were cloned by reverse transcription of RNA from
12 Ebola-infected Vero E6 cells and subsequent
13 amplification of viral cDNAs using the polymerase
14 chain reaction. First strand synthesis was primed with
15 oligo dT (Life Technologies). Second strand synthesis
16 and subsequent amplification of viral cDNAs were
17 performed with gene-specific primers (SEQ ID NOS:8-
18 16). The primer sequences were derived from the
19 GenBank deposited sequences and were designed to
20 contain a ClaI restriction site for cloning the
21 amplified VP genes into the ClaI site of the replicon
22 vector. The letters and numbers in bold print indicate
23 Ebola gene sequences in the primers and the
24 corresponding location numbers based on the GenBank
25 deposited sequences.

26 VP24: (1) forward primer is

27 5'-GGGATCGATCTCCAGACACCAAGCAAGACC-3' (SEQ ID NO:8)

28 (10,311-10,331)

29 (2) reverse primer is

30 5'-GGGATCGATGAGTCAGCATATATGAGTTAGCTC-3' (SEQ ID
31 NO:9)

32 (11,122-11,145)

33 VP30: (1) forward primer is

34 5'-CCCATCGATCAGATCTGCGAACCGGTAGAG-3' SEQ ID NO:10)

35 (8408-8430)

36 (2) reverse primer is

37 5'-CCCATCGATGTACCCTCATCAGACCATGAGC-3' (SEQ ID

38 NO:11)

1 (9347-9368)

2 VP35: (1) forward primer is

3 5'-GGGATCGATAGAAAAGCTGGTCTAACAAGATGA-3' (SEQ ID
4 NO:12)

5 (3110-3133)

6 (2) reverse primer is

7 5'-CCCATCGATCTCACAAGTGTATCATTAATGTAACGT-3' (SEQ ID -
8 NO:13) (4218-4244)

9 VP40: (1) forward primer is

10 5'-CCCATCGATCCTACCTCGGCTGAGAGAGTG-3' (SEQ ID NO:14)
11 (4408-4428)

12 (2) reverse primer is

13 5'-CCCATCGATATGTTATGCACTATCCCTGAGAAG-3' (SEQ ID
14 NO:15)

15 (5495-5518)

16 VP30 #2:

17 (1) forward primer as for VP30 above

18 (2) reverse primer is

19 5'-CCCATCGATCTGTTAGGGTTGTATCATACC-3' (SEQ ID NO:16)

20

21 The Ebola virus genes cloned into the VEE
22 replicon were sequenced. Changes in the DNA sequence
23 relative to the sequence published by Sanchez et al.
24 (1993) are described relative to the nucleotide (nt)
25 sequence number from GenBank (accession number
26 L11365).

27 The nucleotide sequence we obtained for Ebola
28 virus GP (SEQ ID NO:1) differed from the GenBank
29 sequence by a transition from A to G at nt 8023. This
30 resulted in a change in the amino acid sequence from
31 Ile to Val at position 662 (SEQ ID NO: 17).

32 The nucleotide sequence we obtained for Ebola
33 virus NP (SEQ ID NO:2) differed from the GenBank
34 sequence at the following 4 positions: insertion of a
35 C residue between nt 973 and 974, deletion of a G
36 residue at nt 979, transition from C to T at nt 1307,
37 and a transversion from A to C at nt 2745. These
38 changes resulted in a change in the protein sequence

1 from Arg to Glu at position 170 and a change from Leu
2 to Phe at position 280 (SEQ ID NO: 18).

3 The Ebola virus VP24 nucleotide sequence (SEQ ID
4 NO:3) differed from the GenBank sequence at 6
5 positions, resulting in 3 nonconservative changes in
6 the amino acid sequence. The changes in the DNA
7 sequence of VP24 consisted of a transversion from G to
8 C at nt 10795, a transversion from C to G at nt 10796,
9 a transversion from T to A at nt 10846, a transversion
10 from A to T at nt 10847, a transversion from C to G at
11 nt 11040, and a transversion from C to G at nt 11041.
12 The changes in the amino acid sequence of VP24
13 consisted of a Cys to Ser change at position 151, a
14 Leu to His change at position 168, and a Pro to Gly
15 change at position 233 (SEQ ID NO: 19).

16 Two different sequences for the Ebola virus VP30
17 gene, VP30 and VP30#2 (SEQ ID NOS: 4 and 7) are
18 included. Both of these sequences differ from the
19 GenBank sequence by the insertion of an A residue in
20 the upstream noncoding sequence between nt 8469 and
21 8470 and an insertion of a T residue between nt 9275
22 and 9276 that results in a change in the open reading
23 frame of VP30 and VP30#2 after position 255 (SEQ ID
24 NOS: 20 and 23). As a result, the C-terminus of the
25 VP30 protein differs significantly from that
26 previously reported. In addition to these 2 changes,
27 the VP30#2 nucleic acid in SEQ ID NO:7 contains a
28 conservative transition from T to C at nt 9217.
29 Because the primers originally used to clone the VP30
30 gene into the replicon were designed based on the
31 GenBank sequence, the first clone that we constructed
32 (SEQ ID NO: 4) did not contain what we believe to be
33 the authentic C-terminus of the protein. Therefore,
34 in the absence of the VP30 stop codon, the C-terminal
35 codon was replaced with 37 amino acids derived from
36 the vector sequence. The resulting VP30 construct
37 therefore differed from the GenBank sequence in that
38 it contained 32 amino acids of VP30 sequence

1 (positions 256 to 287, SEQ ID NO:20) and 37 amino
2 acids of irrelevant sequence (positions 288 to 324,
3 SEQ ID NO:20) in the place of the C-terminal 5 amino
4 acids reported in GenBank. However, inclusion of 37
5 amino acids of vector sequence in place of the C-
6 terminal amino acid (Pro, SEQ ID NO: 23) did not
7 inhibit the ability of the protein to serve as a
8 protective antigen in BALB/c mice. We are currently
9 examining the ability of the new VEE replicon
10 construct, which we believe contains the authentic C-
11 terminus of VP30 (VP30#2, SEQ ID NO: 23), to protect
12 mice against a lethal Ebola challenge.

13 The nucleotide sequence for Ebola virus VP35 (SEQ
14 ID NO:5) differed from the GenBank sequence by a
15 transition from T to C at nt 4006, a transition from T
16 to C at nt 4025, and an insertion of a T residue
17 between nt 4102 and 4103. These sequence changes
18 resulted in a change from a Ser to a Pro at position
19 293 and a change from Phe to Ser at position 299 (SEQ
20 ID NO: 21). The insertion of the T residue resulted
21 in a change in the open reading frame of VP35 from
22 that previously reported by Sanchez *et al.* (1993)
23 following amino acid number 324. As a result, Ebola
24 virus VP35 encodes a protein of 340 amino acids, where
25 amino acids 325 to 340 (SEQ ID NO: 21) differ from and
26 replace the C-terminal 27 amino acids of the
27 previously published sequence.

28 Sequencing of VP30 and VP35 was also performed
29 on RT/PCR products from RNA derived from cells that
30 were infected with Ebola virus 1976, Ebola virus 1995
31 or the mouse-adapted Ebola virus. The changes noted
32 above for the Vrep constructs were also found in these
33 Ebola viruses. Thus, we believe that these changes are
34 real events and not artifacts of cloning.

35 The Ebola virus VP40 nucleotide sequence (SEQ ID
36 NO:6) differed from the GenBank sequence by a
37 transversion from a C to G at nt 4451 and a transition
38 from a G to A at nt 5081. These sequence changes did

1 not alter the protein sequence of VP40 (SEQ ID NO: 22)
2 from that of the published sequence.

3 DNA or polynucleotide sequences to which the
4 invention also relates include sequences of at least
5 about 6 nucleotides, preferably at least about 8
6 nucleotides, more preferably at least about 10-12
7 nucleotides, most preferably at least about 15-20
8 nucleotides corresponding, i.e., homologous to or
9 complementary to, a region of the Ebola nucleotide
10 sequences described above. Preferably, the sequence of
11 the region from which the polynucleotide is derived is
12 homologous to or complementary to a sequence which is
13 unique to the Ebola genes. Whether or not a sequence is
14 unique to the Ebola gene can be determined by techniques
15 known to those of skill in the art. For example, the
16 sequence can be compared to sequences in databanks,
17 e.g., GenBank and compared by DNA:DNA hybridization.
18 Regions from which typical DNA sequences may be derived
19 include but are not limited to, for example, regions
20 encoding specific epitopes, as well as non-transcribed
21 and/or non-translated regions.

22 The derived polynucleotide is not necessarily
23 physically derived from the nucleotide sequences shown
24 in SEQ ID NO:1-7, but may be generated in any manner,
25 including for example, chemical synthesis or DNA
26 replication or reverse transcription or transcription,
27 which are based on the information provided by the
28 sequence of bases in the region(s) from which the
29 polynucleotide is derived. In addition, combinations
30 of regions corresponding to that of the designated
31 sequence may be modified in ways known in the art to
32 be consistent with an intended use. The sequences of
33 the present invention can be used in diagnostic assays
34 such as hybridization assays and polymerase chain
35 reaction assays, for example, for the discovery of
36 other Ebola sequences.

37 In another embodiment, the present invention
38 relates to a recombinant DNA molecule that includes a

1 vector and a DNA sequence as described above. The
2 vector can take the form of a plasmid, a eukaryotic
3 expression vector such as pcDNA3.1, pRcCMV2,
4 pZeoSV2, or pCDM8, which are available from Invitrogen,
5 or a virus vector such as baculovirus vectors,
6 retrovirus vectors or adenovirus vectors, alphavirus
7 vectors, and others known in the art.

8 In a further embodiment, the present invention
9 relates to host cells stably transformed or
10 transfected with the above-described recombinant DNA
11 constructs. The host cell can be prokaryotic (for
12 example, bacterial), lower eukaryotic (for example,
13 yeast or insect) or higher eukaryotic (for example,
14 all mammals, including but not limited to mouse and
15 human). Both prokaryotic and eukaryotic host cells may
16 be used for expression of the desired coding sequences
17 when appropriate control sequences which are
18 compatible with the designated host are used.

19 Among prokaryotic hosts, *E. coli* is the most
20 frequently used host cell for expression. General
21 control sequences for prokaryotes include promoters
22 and ribosome binding sites. Transfer vectors
23 compatible with prokaryotic hosts are commonly derived
24 from a plasmid containing genes conferring ampicillin
25 and tetracycline resistance (for example, pBR322) or
26 from the various pUC vectors, which also contain
27 sequences conferring antibiotic resistance. These
28 antibiotic resistance genes may be used to obtain
29 successful transformants by selection on medium
30 containing the appropriate antibiotics. Please see
31 e.g., Maniatis, Fritsch and Sambrook, Molecular
32 Cloning; A Laboratory Manual (1982) or DNA Cloning,
33 Volumes I and II (D. N. Glover ed. 1985) for general
34 cloning methods. The DNA sequence can be present in
35 the vector operably linked to sequences encoding an
36 IgG molecule, an adjuvant, a carrier, or an agent for

1 aid in purification of Ebola proteins, such as
2 glutathione S-transferase.

3 In addition, the Ebola virus gene products can
4 also be expressed in eukaryotic host cells such as
5 yeast cells and mammalian cells. *Saccharomyces*
6 *cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia*
7 *pastoris* are the most commonly used yeast hosts.
8 Control sequences for yeast vectors are known in the
9 art. Mammalian cell lines available as hosts for
10 expression of cloned genes are known in the art and
11 include many immortalized cell lines available from
12 the American Type Culture Collection (ATCC), such as
13 CHO cells, Vero cells, baby hamster kidney (BHK) cells
14 and COS cells, to name a few. Suitable promoters are
15 also known in the art and include viral promoters such
16 as that from SV40, Rous sarcoma virus (RSV),
17 adenovirus (ADV), bovine papilloma virus (BPV), and
18 cytomegalovirus (CMV). Mammalian cells may also
19 require terminator sequences, poly A addition
20 sequences, enhancer sequences which increase
21 expression, or sequences which cause amplification of
22 the gene. These sequences are known in the art.

23 The transformed or transfected host cells can be
24 used as a source of DNA sequences described above.
25 When the recombinant molecule takes the form of an
26 expression system, the transformed or transfected
27 cells can be used as a source of the protein described
28 below.

29 In another embodiment, the present invention
30 relates to Ebola virion proteins such as GP having an
31 amino acid sequence corresponding to SEQ ID NO:17
32 encompassing 676 amino acids, NP, having an amino acid
33 sequence corresponding to SEQ ID NO:18 encompassing
34 739 amino acids, VP24, having an amino acid sequence
35 corresponding to SEQ ID NO:19 encompassing 251 amino
36 acids, VP30, having an amino acid sequence
37 corresponding to SEQ ID NO:20 encompassing 324 amino
38 acids, VP35, having an amino acid sequence

1 corresponding to SEQ ID NO:21 encompassing 340 amino
2 acids, and VP40, having an amino acid sequence
3 corresponding to SEQ ID NO:22, encompassing 326 amino
4 acids, and VP30#2, having an amino acid sequence
5 corresponding to SEQ ID NO:23 encompassing 288 amino
6 acids, or any allelic variation of the amino acid
7 sequences. By allelic variation is meant a natural or
8 synthetic change in one or more amino acids which
9 occurs between different serotypes or strains of Ebola
10 virus and does not affect the antigenic properties of
11 the protein. There are different strains of Ebola
12 (Zaire 1976, Zaire 1995, Reston, Sudan, and Ivory
13 Coast). The NP and VP genes of these different viruses
14 have not been sequenced. It would be expected that
15 these proteins would have homology among different
16 strains and that vaccination against one Ebola virus
17 strain might afford cross protection to other Ebola
18 virus strains.

19 A polypeptide or amino acid sequence derived
20 from any of the amino acid sequences in SEQ ID NO:17,
21 18, 19, 20, 21, 22, and 23 refers to a polypeptide
22 having an amino acid sequence identical to that of a
23 polypeptide encoded in the sequence, or a portion
24 thereof wherein the portion consists of at least 2-5
25 amino acids, preferably at least 8-10 amino acids, and
26 more preferably at least 11-15 amino acids, or which
27 is immunologically identifiable with a polypeptide
28 encoded in the sequence.

29 A recombinant or derived polypeptide is not
30 necessarily translated from a designated nucleic acid
31 sequence, or the DNA sequence found in GenBank
32 accession number L11365. It may be generated in any
33 manner, including for example, chemical synthesis, or
34 expression from a recombinant expression system.

35 When the DNA or RNA sequences described above
36 are in a replicon expression system, such as the VEE
37 replicon described above, the proteins can be
38 expressed *in vivo*. The DNA sequence for any of the

1 GP, NP, VP24, VP30, VP35, and VP40 virion proteins can
2 be cloned into the multiple cloning site of a replicon
3 such that transcription of the RNA from the replicon
4 yields an infectious RNA encoding the Ebola protein or
5 proteins of interest (see Figure 2A, 2B and 2C). The
6 replicon constructs include Ebola virus GP (SEQ ID
7 NO:1) cloned into a VEE replicon (VRepEboGP), Ebola
8 virus NP (SEQ ID NO:2) cloned into a VEE replicon
9 (VRepEboNP), Ebola virus VP24 (SEQ ID NO:3) cloned
10 into a VEE replicon (VRepEboVP24), Ebola virus VP30
11 (SEQ ID NO:4) or VP30#2 (SEQ ID NO:7) cloned into a
12 VEE replicon (VRepEboVP30 or VRepEboVP30(#2)), Ebola
13 virus VP35 (SEQ ID NO:5) cloned into a VEE replicon
14 (VRepEboVP35), and Ebola virus VP40 (SEQ ID NO:6)
15 cloned into a VEE replicon (VRepEboVP40). The
16 replicon DNA or RNA can be used as a vaccine for
17 inducing protection against infection with Ebola.
18 Use of helper RNAs containing sequences necessary for
19 packaging of the viral replicon transcripts will
20 result in the production of virus-like particles
21 containing replicon RNAs (Figure 3). These packaged
22 replicons will infect host cells and initiate a single
23 round of replication resulting in the expression of
24 the Ebola proteins in said infected cells. The
25 packaged replicon constructs (i.e. VEE virus replicon
26 particles, VRP) include those that express Ebola virus
27 GP (EboGPVRP), Ebola virus NP (EboNPVRP), Ebola virus
28 VP24 (EboVP24VRP), Ebola virus VP30 (EboVP30VRP or
29 EboVP30VRP(#2)), Ebola virus VP35 (EboVP35VRP), and
30 Ebola virus VP40 (EboVP40VRP).

31 In another embodiment, the present invention
32 relates to RNA molecules resulting from the
33 transcription of the constructs described above. The
34 RNA molecules can be prepared by *in vitro* transcription
35 using methods known in the art and described in the
36 Examples below. Alternatively, the RNA molecules can be
37 produced by transcription of the constructs *in vivo*, and
38 isolating the RNA. These and other methods for

1 obtaining RNA transcripts of the constructs are known in
2 the art. Please see Current Protocols in Molecular
3 Biology. Frederick M. Ausubel et al. (eds.), John Wiley
4 and Sons, Inc. The RNA molecules can be used, for
5 example, as a direct RNA vaccine, or to transfect cells
6 along with RNA from helper plasmids, one of which
7 expresses VEE glycoproteins and the other VEE capsid -
8 proteins, as described above, in order to obtain
9 replicon particles.

10 In a further embodiment, the present invention
11 relates to a method of producing the recombinant or
12 fusion protein which includes culturing the above-
13 described host cells under conditions such that the
14 DNA fragment is expressed and the recombinant or
15 fusion protein is produced thereby. The recombinant or
16 fusion protein can then be isolated using methodology
17 well known in the art. The recombinant or fusion
18 protein can be used as a vaccine for immunity against
19 infection with Ebola or as a diagnostic tool for
20 detection of Ebola infection.

21 In another embodiment, the present invention
22 relates to antibodies specific for the above-described
23 recombinant proteins (or polypeptides). For instance,
24 an antibody can be raised against a peptide having the
25 amino acid sequence of any of SEQ ID NO:17-25, or
26 against a portion thereof of at least 10 amino acids,
27 preferably, 11-15 amino acids. Persons with ordinary
28 skill in the art using standard methodology can raise
29 monoclonal and polyclonal antibodies to the protein(or
30 polypeptide) of the present invention, or a unique
31 portion thereof. Materials and methods for producing
32 antibodies are well known in the art (see for example
33 Goding, In Monoclonal Antibodies: Principles and
34 Practice, Chapter 4, 1986).

35 In a further embodiment, the present invention
36 relates to a method of detecting the presence of
37 antibodies against Ebolavirus in a sample. Using

1 standard methodology well known in the art, a
2 diagnostic assay can be constructed by coating on a
3 surface (i.e. a solid support for example, a
4 microtitration plate, a membrane (e.g. nitrocellulose
5 membrane) or a dipstick), all or a unique portion of
6 any of the Ebola proteins described above or any
7 combination thereof, and contacting it with the serum
8 of a person or animal suspected of having Ebola. The
9 presence of a resulting complex formed between the
10 Ebola protein(s) and serum antibodies specific
11 therefor can be detected by any of the known methods
12 common in the art, such as fluorescent antibody
13 spectroscopy or colorimetry. This method of detection
14 can be used, for example, for the diagnosis of Ebola
15 infection and for determining the degree to which an
16 individual has developed virus-specific Abs after
17 administration of a vaccine.

18 In yet another embodiment, the present invention
19 relates to a method for detecting the presence of
20 Ebola virion proteins in a sample. Antibodies against
21 GP, NP, and the VP proteins could be used for
22 diagnostic assays. Using standard methodology well
23 known in the art, a diagnostics assay can be
24 constructed by coating on a surface (i.e. a solid
25 support, for example, a microtitration plate or a
26 membrane (e.g. nitrocellulose membrane)), antibodies
27 specific for any of the Ebola proteins described
28 above, and contacting it with serum or a tissue sample
29 of a person suspected of having Ebola infection. The
30 presence of a resulting complex formed between the
31 protein or proteins in the serum and antibodies
32 specific therefor can be detected by any of the known
33 methods common in the art, such as fluorescent
34 antibody spectroscopy or colorimetry. This method of
35 detection can be used, for example, for the diagnosis
36 of Ebola virus infection.

37 In another embodiment, the present invention
38 relates to a diagnostic kit which contains any

1 combination of the Ebola proteins described above and
2 ancillary reagents that are well known in the art and
3 that are suitable for use in detecting the presence of
4 antibodies to Ebola in serum or a tissue sample.
5 Tissue samples contemplated can be from monkeys,
6 humans, or other mammals.

7 In yet another embodiment, the present invention
8 relates to DNA or nucleotide sequences for use in
9 detecting the presence of Ebola virus using the
10 reverse transcription-polymerase chain reaction (RT-
11 PCR). The DNA sequence of the present invention can
12 be used to design primers which specifically bind to
13 the viral RNA for the purpose of detecting the
14 presence of Ebola virus or for measuring the amount
15 of Ebola virus in a sample. The primers can be any
16 length ranging from 7 to 400 nucleotides, preferably
17 at least 10 to 15 nucleotides, or more preferably 18
18 to 40 nucleotides. Reagents and controls necessary
19 for PCR reactions are well known in the art. The
20 amplified products can then be analyzed for the
21 presence of viral sequences, for example by gel
22 fractionation, with or without hybridization, by
23 radiochemistry, and immunochemistry techniques.

24 In yet another embodiment, the present invention
25 relates to a diagnostic kit which contains PCR primers
26 specific for Ebola virus and ancillary reagents for
27 use in detecting the presence or absence of Ebola in a
28 sample using PCR. Samples contemplated can be obtained
29 from human, animal, e.g., horse, donkey, pig, mouse,
30 hamster, monkey, or other mammals, birds, and insects,
31 such as mosquitoes.

32 In another embodiment, the present invention
33 relates to an Ebola vaccine comprising VRPs that
34 express one or more of the Ebola proteins described
35 above. The vaccine is administered to a subject
36 wherein the replicon is able to initiate one round of
37 replication producing the Ebola proteins to which a

1 protective immune response is initiated in said
2 subject.

3 It is likely that the protection afforded by
4 these genes is due to both the humoral (antibodies
5 (Abs)) and cellular (cytotoxic T cells (CTLs)) arms of
6 the immune system. Protective immunity induced to a
7 specific protein may comprise humoral immunity,
8 cellular immunity, or both. The only Ebola virus
9 protein known to be on the outside of the virion is
10 the GP. The presence of GP on the virion surface
11 makes it a likely target for GP-specific Abs that may
12 bind either extracellular virions or infected cells
13 expressing GP on their surfaces. Serum transfer
14 studies in this invention demonstrate that Abs that
15 recognize GP protect mice against lethal Ebola virus
16 challenge.

17 In contrast, transfer of Abs specific for NP,
18 VP24, VP30, VP35, or VP40 did not protect mice against
19 lethal Ebola challenge. This data, together with the
20 fact that these are internal virion proteins that are
21 not readily accessible to Abs on either extracellular
22 virions or the surface of infected cells, suggest that
23 the protection induced in mice by these proteins is
24 mediated by CTLs.

25 CTLs can bind to and lyse virally infected cells.
26 This process begins when the proteins produced by
27 cells are routinely digested into peptides. Some of
28 these peptides are bound by the class I or class II
29 molecules of the major histocompatibility complex
30 (MHC), which are then transported to the cell surface.
31 During virus infections, viral proteins produced
32 within infected cells also undergo this process. CTLs
33 that have receptors that bind to both a specific
34 peptide and the MHC molecule holding the peptide lyse
35 the peptide-bearing cell, thereby limiting virus
36 replication. Thus, CTLs are characterized as being
37 specific for a particular peptide and restricted to a
38 class I or class II MHC molecule.

1 CTLs may be induced against any of the Ebola
2 virus proteins, as all of the viral proteins are
3 produced and digested within the infected cell. Thus,
4 protection to Ebola virus could involve CTLs against
5 GP, NP, VP24, VP30, VP35, and/or VP40. It is
6 especially noteworthy that the VP proteins varied in
7 their protective efficacy when tested in genetically
8 inbred mice that differ at the MHC locus. This,
9 together with the inability to demonstrate a role for
10 Abs in protection induced by the VP proteins, strongly
11 supports a role for CTLs. These data also suggest
12 that an eventual vaccine candidate may include several
13 Ebola virus proteins, or several CTL epitopes, capable
14 of inducing broad protection in outbred populations
15 (e.g. people). We have identified two sequences
16 recognized by CTLs. They are Ebola virus NP SEQ ID
17 NO:24 and Ebola virus VP24 SEQ ID NO:25. Testing is
18 in progress to identify the role of CTLs in protection
19 induced by each of these Ebola virus proteins and to
20 define the minimal sequence requirements for the
21 protective response. The CTL assay is well known in
22 the art.

23 An eventual vaccine candidate might
24 comprise these CTL sequences and others. These might
25 be delivered as synthetic peptides, or as fusion
26 proteins, alone or co-administered with cytokines
27 and/or adjuvants or carriers safe for human use, e.g.
28 aluminum hydroxide, to increase immunogenicity. In
29 addition, sequences such as ubiquitin can be added to
30 increase antigen processing for more effective CTL
31 responses.

32 In yet another embodiment, the present invention
33 relates to a method for providing immunity against
34 Ebola virus, said method comprising administering one
35 or more VRPs expressing any combination of the GP, NP,
36 VP24, VP30 or VP30#2, VP35 and VP40 Ebola proteins to
37 a subject such that a protective immune reaction is
38 generated.

1 Vaccine formulations of the present invention
2 comprise an immunogenic amount of a VRP, such as for
3 example EboVP24VRP described above, or, for a
4 multivalent vaccine, a combination of replicons, in a
5 pharmaceutically acceptable carrier. An "immunogenic
6 amount" is an amount of the VRP(s) sufficient to evoke
7 an immune response in the subject to which the vaccine-
8 is administered. An amount of from about 10^4 - 10^8
9 focus-forming units per dose is suitable, depending
10 upon the age and species of the subject being treated.
11 The subject may be inoculated 2-3 times. Exemplary
12 pharmaceutically acceptable carriers include, but are
13 not limited to, sterile pyrogen-free water and sterile
14 pyrogen-free physiological saline solution.

15 Administration of the VRPs disclosed herein may
16 be carried out by any suitable means, including
17 parenteral injection (such as intraperitoneal,
18 subcutaneous, or intramuscular injection), *in ovo*
19 injection of birds, orally, or by topical application
20 of the virus (typically carried in a pharmaceutical
21 formulation) to an airway surface. Topical application
22 of the virus to an airway surface can be carried out
23 by intranasal administration (e.g., by use of dropper,
24 swab, or inhaler which deposits a pharmaceutical
25 formulation intranasally). Topical application of the
26 virus to an airway surface can also be carried out by
27 inhalation administration, such as by creating
28 respirable particles of a pharmaceutical formulation
29 (including both solid particles and liquid particles)
30 containing the replicon as an aerosol suspension, and
31 then causing the subject to inhale the respirable
32 particles. Methods and apparatus for administering
33 respirable particles of pharmaceutical formulations
34 are well known, and any conventional technique can be
35 employed. Oral administration may be in the form of
36 an ingestible liquid or solid formulation.

1 When the replicon RNA or DNA is used as a vaccine,
2 the replicon RNA or DNA can be administered directly
3 using techniques such as delivery on gold beads (gene
4 gun), delivery by liposomes, or direct injection, among
5 other methods known to people in the art. Any one or
6 more DNA constructs or replicating RNA described above
7 can be use in any combination effective to elicit an
8 immunogenic response in a subject. Generally, the
9 nucleic acid vaccine administered may be in an amount of
10 about 1-5 ug of nucleic acid per dose and will depend on
11 the subject to be treated, capacity of the subject's
12 immune system to develop the desired immune response,
13 and the degree of protection desired. Precise amounts
14 of the vaccine to be administered may depend on the
15 judgement of the practitioner and may be peculiar to
16 each subject and antigen.

17 The vaccine may be given in a single dose
18 schedule, or preferably a multiple dose schedule in
19 which a primary course of vaccination may be with 1-10
20 separate doses, followed by other doses given at
21 subsequent time intervals required to maintain and or
22 reinforce the immune response, for example, at 1-4
23 months for a second dose, and if needed, a subsequent
24 dose(s) after several months. Examples of suitable
25 immunization schedules include: (i) 0, 1 months and 6
26 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1
27 month, (iv) 0 and 6 months, or other schedules
28 sufficient to elicit the desired immune responses
29 expected to confer protective immunity, or reduce
30 disease symptoms, or reduce severity of disease.

31 The following examples are included to demonstrate
32 preferred embodiments of the invention. It should be
33 appreciated by those of skill in the art that the
34 techniques disclosed in the examples which follow
35 represent techniques discovered by the inventors and
36 thought to function well in the practice of the
37 invention, and thus can be considered to constitute
38 preferred modes for its practice. However, those of

1 skill in the art should, in light of the present
2 disclosure, appreciate that many changes can be made in
3 the specific embodiments which are disclosed and still
4 obtain a like or similar result without departing from
5 the spirit and scope of the invention.

6

7 The following MATERIALS AND METHODS were used in -
8 the examples that follow.

9 Cells lines and viruses

10 BHK (ATCC CCL 10), Vero 76 (ATCC CRL 1587), and
11 Vero E6 (ATCC CRL 1586) cell lines were maintained in
12 minimal essential medium with Earle's salts, 5-10%
13 fetal bovine serum, and 50 µg/mL gentamicin sulfate.

14 For CTL assays, EL4 (ATCC TIB39), L5178Y (ATCC CRL
15 1723) and P815 (ATCC TIB64) were maintained in
16 Dulbecco's minimal essential medium supplemented with
17 5-10% fetal bovine serum and antibiotics.

18 A stock of the Zaire strain of Ebola virus
19 originally isolated from a patient in the 1976
20 outbreak (Mayinga) and passaged intracerebrally 3
21 times in suckling mice and 2 times in Vero cells was
22 adapted to adult mice through serial passage in
23 progressively older suckling mice (Bray et al., (1998)
24 *J. Infect. Dis.* **178**, 651-661). A plaque-purified
25 ninth-mouse-passage isolate which was uniformly lethal
26 for adult mice ("mouse-adapted virus") was propagated
27 in Vero E6 cells, aliquotted, and used in all mouse
28 challenge experiments and neutralization assays.

29 A stock of the Zaire strain of Ebola 1976 virus
30 was passaged spleen to spleen in strain 13 guinea pigs
31 four times. This guinea pig-adapted strain was used
32 to challenge guinea pigs.

33 Construction and packaging of recombinant VEE
34 virus replicons (VRPs)

35 Replicon RNAs were packaged into VRPs as
36 described (Pushko et al., 1997, supra). Briefly,
37 capped replicon RNAs were produced *in vitro* by T7 run-

1 off transcription of NotI-digested plasmid templates
2 using the RiboMAX T7 RNA polymerase kit (Promega).
3 BHK cells were co-transfected with the replicon RNAs
4 and the 2 helper RNAs expressing the structural
5 proteins of the VEE virus. The cell culture
6 supernatants were harvested approximately 30 hours
7 after transfection and the replicon particles were
8 concentrated and purified by centrifugation through a
9 20% sucrose cushion. The pellets containing the
10 packaged replicon particles were suspended in PBS and
11 the titers were determined by infecting Vero cells
12 with serial dilutions of the replicon particles and
13 enumerating the infected cells by indirect
14 immunofluorescence with antibodies specific for the
15 Ebola proteins.

16 Immunoprecipitation of Ebola virus proteins
17 expressed from VEE virus replicons

18 BHK cells were transfected with either the Ebola
19 virus GP, NP, VP24, VP30, VP35, or VP40 replicon RNAs.
20 At 24 h post-transfection, the culture medium was
21 replaced with minimal medium lacking cysteine and
22 methionine, and proteins were labeled for 1 h with
23 ³⁵S-labeled methionine and cysteine. Cell lysates or
24 supernatants (supe) were collected and
25 immunoprecipitated with polyclonal rabbit anti-Ebola
26 virus serum bound to protein A beads. ³⁵S-labeled
27 Ebola virus structural proteins from virions grown in
28 Vero E6 cells were also immunoprecipitated as a
29 control for each of the virion proteins.
30 Immunoprecipitated proteins were resolved by
31 electrophoresis on an 11% SDS-polyacrylamide gel and
32 were visualized by autoradiography.

33 Vaccination of Mice With VEE Virus Replicons

34 Groups of 10 BALB/c or C57BL/6 mice per experiment
35 were subcutaneously injected at the base of the neck
36 with 2×10^6 focus-forming units of VRPs encoding the
37 Ebola virus genes. As controls, mice were also

1 injected with either a control VRP encoding the Lassa
2 nucleoprotein (NP) or with PBS. For booster
3 inoculations, animals received identical injections at
4 1 month intervals. Data are recorded as the combined
5 results of 2 or 3 separate experiments.

6 Ebola Infection of Mice

7 One month after the final booster inoculation,
8 mice were transferred to a BSL-4 containment area and
9 challenged by intraperitoneal (ip) inoculation of 10
10 plaque-forming units (pfu) of mouse-adapted Ebola
11 virus (approximately 300 times the dose lethal for 50%
12 of adult mice). The mice were observed daily, and
13 morbidity and mortality were recorded. Animals
14 surviving at day 21 post-infection were injected again
15 with the same dose of virus and observed for another
16 21 days.

17 In some experiments, 4 or 5 mice from vaccinated
18 and control groups were anesthetized and exsanguinated
19 on day 4 (BALB/c mice) or day 5 (C57BL/6 mice)
20 following the initial viral challenge. The viral
21 titers in individual sera were determined by plaque
22 assay.

23 Passive Transfer Of Immune Sera to Naive Mice.

24 Donor sera were obtained 28 days after the third
25 inoculation with 2×10^6 focus-forming units of VRPs
26 encoding the indicated Ebola virus gene, the control
27 Lassa NP gene, or from unvaccinated control mice. One
28 mL of pooled donor sera was administered
29 intraperitoneally (ip) to naive, syngeneic mice 24 h
30 prior to intraperitoneal challenge with 10 pfu of
31 mouse-adapted Ebola virus.

32 Vaccination and Challenge of Guinea Pigs.

33 EboGPVRP or EboNPVRP (1×10^7 focus-forming units
34 in 0.5ml PBS) were administered subcutaneously to
35 inbred strain 2 or strain 13 guinea pigs (300-400g).
36 Groups of five guinea pigs were inoculated on days 0
37 and 28 at one (strain 2) or two (strain 13) dorsal

1 sites. Strain 13 guinea pigs were also boosted on day
2 126. One group of Strain 13 guinea pigs was
3 vaccinated with both the GP and NP constructs. Blood
4 samples were obtained after vaccination and after
5 viral challenge. Guinea pigs were challenged on day
6 56 (strain 2) or day 160 (strain 13) by subcutaneous
7 administration of 1000 LD₅₀ (1×10^4 PFU) of guinea
8 pig-adapted Ebola virus. Animals were observed daily
9 for 60 days, and morbidity (determined as changes in
10 behavior, appearance, and weight) and survival were
11 recorded. Blood samples were taken on the days
12 indicated after challenge and viremia levels were
13 determined by plaque assay.

14 Virus titration and neutralization assay. Viral
15 stocks were serially diluted in growth medium,
16 adsorbed onto confluent Vero E6 cells in 6- or 12-well
17 dishes, incubated for 1 hour at 37°C, and covered with
18 an agarose overlay (Moe, J. et al. (1981) *J. Clin.*
19 *Microbiol.* 13:791-793). A second overlay containing 5%
20 neutral red solution in PBS or agarose was added 6
21 days later, and plaques were counted the following
22 day. Pooled pre-challenge serum samples from some of
23 the immunized groups were tested for the presence of
24 Ebola-neutralizing antibodies by plaque reduction
25 neutralization assay. Aliquots of Ebola virus in
26 growth medium were mixed with serial dilutions of test
27 serum, or with normal serum, or medium only, incubated
28 at 37°C for 1 h, and used to infect Vero E6 cells.
29 Plaques were counted 1 week later.

30 Cytotoxic T cell assays. BALB/c and C57BL/6 mice
31 were inoculated with VRPs encoding Ebola virus NP or
32 VP24 or the control Lassa NP protein. Mice were
33 euthanized at various times after the last inoculation
34 and their spleens removed. The spleens were gently
35 ruptured to generate single cell suspensions. Spleen
36 cells (1×10^6 / ml) were cultured *in vitro* for 2 days
37 in the presence of 10-25 μ M of peptides synthesized

1 from Ebola virus NP or VP24 amino acid sequences, and
2 then for an additional 5 days in the presence of
3 peptide and 10% supernatant from concanavalin A-
4 stimulated syngeneic spleen cells. Synthetic peptides
5 were made from Ebola virus amino acid sequences
6 predicted by a computer algorithm (HLA Peptide Binding
7 Predictions, Parker, K. C., et al. (1994) *J. Immunol.*
8 **152**:163) to have a likelihood of meeting the MHC
9 class I binding requirements of the BALB/c (H-2^d) and
10 C57BL/6 (H-2^b) haplotypes. Only 2 of 8 peptides
11 predicted by the algorithm and tested to date have
12 been identified as containing CTL epitopes. After *in*
13 vitro restimulation, the spleen cells were tested in a
14 standard ⁵¹chromium-release assay well known in the
15 art (see, for example, Hart et al. (1991) *Proc. Natl.*
16 *Acad. Sci. USA* **88**: 9449-9452). Percent specific lysis
17 of peptide-coated, MHC-matched or mismatched target
18 cells was calculated as:

19

20
$$\frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100$$

21

22

23 Spontaneous cpm are the number of counts
24 released from target cells incubated in medium.
25 Maximum cpm are obtained by lysing target cells with
26 1% Triton X-100. Experimental cpm are the counts from
27 wells in which target cells are incubated with varying
28 numbers of effector (CTL) cells. Target cells tested
29 were L5178Y lymphoma or P815 mastocytoma cells (MHC
30 matched to the H2^d BALB/c mice and EL4 lymphoma cells
31 (MHC matched to the H2^b C57BL/6 mice). The
32 effector:target (E:T) ratios tested were 25:1, 12:1,
33 6:1 and 3:1.

34

EXAMPLE 1

35 Survival Of Mice Inoculated With VRPs Encoding36 Ebola Proteins. Mice were inoculated two or three37 times at 1 month intervals with 2×10^6 focus-forming

1 units of VRPs encoding individual Ebola virus genes,
 2 or Lassa virus NP as a control, or with phosphate
 3 buffered saline (PBS). Mice were challenged with 10
 4 pfu of mouse-adapted Ebola virus one month after the
 5 final immunization. The mice were observed daily, and
 6 morbidity and mortality data are shown in Table 1A for
 7 BALB/c mice and Table 1B for C57BL/6 mice. The viral
 8 titers in individual sera of some mice on day 4
 9 (BALB/c mice) or day 5 (C57BL/6 mice) following the
 10 initial viral challenge were determined by plaque
 11 assay.

12
 13 **Table 1. Survival Of Mice Inoculated With VRPs**
 14 **Encoding Ebola Proteins**

15 **A. BALB/c Mice**

16	VRP	#Injections	S/T ¹ (%)	MDD ²	V/T ³	Viremia ⁴
17	EboNP	3	30/30 (100%)	5/5	5.2	
18		2	19/20 (95%)	7	5/5	4.6
19						
20	EboGP	3	15/29 (52%)	8	1/5	6.6
21		2	14/20 (70%)	7	3/5	3.1
22						
23	EboVP24	3	27/30 (90%)	8	5/5	5.2
24		2	19/20 (95%)	6	4/4	4.8
25						
26	EboVP30	3	17/20 (85%)	7	5/5	6.2
27		2	11/20 (55%)	7	5/5	6.5
28						
29	EboVP35	3	5/19 (26%)	7	5/5	6.9
30		2	4/20 (20%)	7	5/5	6.5
31						
32	EboVP40	3	14/20 (70%)	8	5/5	4.6
33		2	17/20 (85%)	7	5/5	5.6
34						
35	LassaNP	3	0/29 (0%)	7	5/5	8.0
36		2	0/20 (0%)	7	5/5	8.4
37						

1	none (PBS)	3	1/30 (3%)	6	5/5	8.3
2		2	0/20 (0%)	6	5/5	8.7
3						
4	B. C57BL/6 Mice					
5						
6	<u>VRP</u>	<u>#Injections</u>	<u>S/T¹ (%)</u>	<u>MDD²</u>	<u>V/T³</u>	<u>Viremia⁴</u>
7						
8	EboNP	3	15/20 (75%)	8	5/5	4.1
9		2	8/10 (80%)	9	ND ⁵	ND
10						
11	EboGP	3	19/20 (95%)	10	0/5	--
12		2	10/10 (100%)	-	ND	ND
13						
14	EboVP24	3	0/20 (0%)	7	5/5	8.6
15						
16	EboVP30	3	2/20 (10%)	8	5/5	7.7
17						
18	EboVP35	3	14/20 (70%)	8	5/5	4.5
19						
20	EboVP40	3	1/20 (5%)	7	4/4	7.8
21						
22	LassaNP	3	1/20 (5%)	7	4/4	8.6
23		2	0/10 (0%)	7	ND	ND
24						
25	none (PBS)	3	3/20 (15%)	7	5/5	8.6
26		2	0/10 (0%)	7	ND	ND

28 ¹S/T, Survivors/total challenged.

29 ²MDD, Mean day to death

30 ³V/T, Number of mice with viremia/total number tested.

31 ⁴Geometric mean of Log₁₀ viremia titers in PFU/mL. Standard
 32 errors for all groups were 1.5 or less, except for the group of
 33 BALB/c mice given 2 inoculations of EboGP, which was 2.2.

34 ⁵ND, not determined.

35

36

37

EXAMPLE 2**VP24-Immunized BALB/c Mice Survive A High-Dose Challenge With Ebola virus.**

BALB/c mice were inoculated two times with 2×10^6 focus-forming units of EboVP24VRP. Mice were challenged with either 1×10^3 pfu or 1×10^5 pfu of mouse-adapted Ebola virus 1 month after the second inoculation. Morbidity and mortality data for these mice are shown in Table 2.

Table 2. VP24-Immunized BALB/c Mice Survive A High-Dose Challenge With Ebola virus

<u>Replicon</u>	<u>Challenge Dose</u>	<u>Survivors/Total</u>
EboVP24	1×10^3 pfu (3×10^4 LD ₅₀)	5/5
EboVP24	1×10^5 pfu (3×10^6 LD ₅₀)	5/5
None	1×10^3 pfu (3×10^4 LD ₅₀)	0/4
None	1×10^5 pfu (3×10^6 LD ₅₀)	0/3

EXAMPLE 3**Passive Transfer Of Immune Sera Can Protect Naive Mice From A Lethal Challenge Of Ebola Virus.**

Donor sera were obtained 28 days after the third inoculation with 2×10^6 focus-forming units of VRPs encoding the indicated Ebola virus gene, the control Lassa NP gene, or from unvaccinated control mice. One mL of pooled donor sera was administered

1 intraperitoneally (ip) to naive, syngeneic mice 24 h
 2 prior to intraperitoneal challenge with 10 pfu of
 3 mouse-adapted Ebola virus.

4

5 **Table 3.** Passive Transfer of Immune Sera Can Protect
 6 Unvaccinated Mice from a Lethal Challenge of Ebola
 7 Virus

8

9 A. BALB/c Mice

10 Specificity of	Survivors	Mean Day
11 <u>Donor Sera</u>	<u>/Total</u>	<u>of Death</u>
12 Ebola GP	15/20	8
13 Ebola NP	1/20	7
14 Ebola VP24	0/20	6
15 Ebola VP30	0/20	7
16 Ebola VP35	ND ¹	ND
17 Ebola VP40	0/20	6
18 Lassa NP	0/20	7
19 Normal mouse sera	0/20	6

20

21 B. C57BL/6 Mice

22 Specificity of	Survivors	Mean Day
23 <u>Donor Sera</u>	<u>/Total</u>	<u>of Death</u>
24 Ebola GP	17/20	7
25 Ebola NP	0/20	7
26 Ebola VP24	ND	ND
27 Ebola VP30	ND	ND
28 Ebola VP35	0/20	7
29 Ebola VP40	ND	ND
30 Lassa NP	0/20	7
31 Normal mouse sera	0/20	7

32

33 ¹ND, not determined

34

35

36

37

EXAMPLE 4**Immunogenicity and Efficacy of VRepEboGP and VRepEboNP in Guinea Pigs.**

EboGPVRP or EboNPVRP (1×10^7 IU in 0.5ml PBS) were administered subcutaneously to inbred strain 2 or strain 13 guinea pigs (300-400g). Groups of five guinea pigs were inoculated on days 0 and 28 at one (strain 2) or two (strain 13) dorsal sites. Strain 13 guinea pigs were also boosted on day 126. One group of Strain 13 guinea pigs was vaccinated with both the GP and NP constructs. Blood samples were obtained after vaccination and after viral challenge.

Sera from vaccinated animals were assayed for antibodies to Ebola by plaque-reduction neutralization, and ELISA. Vaccination with VRepEboGP or NP induced high titers of antibodies to the Ebola proteins (Table 4) in both guinea pig strains. Neutralizing antibody responses were only detected in animals vaccinated with the GP construct (Table 4).

Guinea pigs were challenged on day 56 (strain 2) or day 160 (strain 13) by subcutaneous administration of 1000 LD₅₀ (10^4 PFU) of guinea pig-adapted Ebola virus. Animals were observed daily for 60 days, and morbidity (determined as changes in behavior, appearance, and weight) and survival were recorded. Blood samples were taken on the days indicated after challenge and viremia levels were determined by plaque assay. Strain 13 guinea pigs vaccinated with the GP construct, alone or in combination with NP, survived lethal Ebola challenge (Table 4). Likewise, vaccination of strain 2 inbred guinea pigs with the GP construct protected 3/5 animals against death from lethal Ebola challenge, and significantly prolonged the mean day of death (MDD) in one of the two animals that died (Table 4). Vaccination with NP alone did not protect either guinea pig strain.

Table 4. Immunogenicity and efficacy of VRepEboGP and VRepEboNP in guinea pigs

A. Strain 2 guinea pigs

VRP	ELISA ^a	PRNT ₅₀	Survivors/		Viremia ^c	
			total (MDD ^b)		d7	d14
GP	4.1	30	3/5	(13+2.8)	2.3	1.8
NP	3.9	<10	0/5	(9.2+1.1)	3.0	--
Mock	<1.5	<10	0/5	(8.8+0.5)	3.9	--

B. Strain 13 guinea pigs

VRP	ELISA ^a	PRNT ₅₀	Survivors/		Viremia ^c	
			total (MDD ^b)		d7	d14
GP	4.0	140	5/5		<2.0	<2.0
GP/NP	3.8	70	5/5		<2.0	<2.0
NP	2.8	<10	1/5	(8.3+2.2)	4.6	--
Lassa NP	<1.5	<10	2/5	(8.3+0.6)	4.8	--

^aData are expressed as geometric mean titers, log₁₀.

^bMDD, mean day to death

^cGeometric mean of log₁₀ viremia titers in PFU/mL. Standard errors for all groups were 0.9 or less.

EXAMPLE 5

Induction of murine CTL responses to Ebola virus NP and Ebola virus VP24 proteins.

BALB/c and C57BL/6 mice were inoculated with VRPs encoding Ebola virus NP or VP24. Mice were euthanized at various times after the last inoculation and their spleens removed. Spleen cells (1×10^6 /ml) were cultured *in vitro* for 2 days in the presence of 10 to 25 μ M of peptides, and then for an additional 5 days in the presence of peptide and 10% supernatant from concanavalin A-stimulated syngeneic spleen cells. After *in vitro* restimulation, the spleen cells were tested in a standard ⁵¹chromium-release assay. Percent specific lysis of peptide-coated, MHC-matched or mismatched target cells was calculated as:

1

2 Experimental cpm- Spontaneous cpm x 100

3 Maximum cpm-Spontaneous cpm

4

5 In the experiments shown, spontaneous release did not
6 exceed 15%.

7

8 **Table 5.** Induction of murine CTL responses to Ebola
9 virus NP and Ebola virus VP24 proteins.

		% Specific Lysis	
		E:T ratio	
<u>Mice, VRP¹</u>	<u>Peptide²</u>	<u>Cell³</u>	<u>25</u>
BALB/c, VP24	None	P815	55
BALB/c, VP24	SEQ ID NO:25	P815	93
C57BL/6, EboNP	None	EL4	2
C57BL/6, EboNP ⁴	SEQ ID NO:24	EL4	70
C57BL/6, EboNP	Lassa NP	EL4	2
C57BL/6, LassaNP	None	L5178Y	1
C57BL/6, LassaNP	SEQ ID NO:24	L5178Y	0
C57BL/6, LassaNP	None	EL4	2
C57BL/6, LassaNP	SEQ ID NO:24	EL4	6

22 ¹ Indicates the mouse strain used and the VRP used as the *in*
23 *vivo* immunogen. *In vitro* restimulation was performed using SEQ
24 ID NO:24 peptide for BALB/c mice and SEQ ID NO:23 for all
25 C57BL/6 mice shown.

26 ² Indicates the peptide used to coat the target cells for the
27 chromium release assay.

28 ³ Target cells are MHC-matched to the effector cells, except
29 for the L5178Y cells that are C57BL/6 mismatched.

30 ⁴ High levels of specific lysis (>40%) were also observed using
31 E:T ratios of 12, 6, 3, or 1:1.

32 **RESULTS AND DISCUSSION**

33 Ebola Zaire 1976 (Mayinga) virus causes acute
34 hemorrhagic fever characterized by high mortality.
35 There are no current vaccines or effective therapeutic
36 measures to protect individuals who are exposed to
37 this virus. In addition, it is not known which genes

1 are essential for evoking protective immunity and
2 should therefore be included in a vaccine designed for
3 human use. In this study, the GP, NP, VP24, VP30,
4 VP35, and VP40 virion protein genes of the Ebola Zaire
5 1976 (Mayinga) virus were cloned and inserted into a
6 Venezuelan equine encephalitis (VEE) virus replicon
7 vector (VRep) as shown in Figure 2A and 2B. These
8 VReps were packaged as VEE replicon particles (VRPs)
9 using the VEE virus structural proteins provided as
10 helper RNAs, as shown in Figure 3. This enables
11 expression of the Ebola virus proteins in host cells.
12 The Ebola virus proteins produced from these
13 constructs were characterized *in vitro* and were shown
14 to react with polyclonal rabbit anti-Ebola virus
15 antibodies bound to Protein A beads following SDS gel
16 electrophoresis of immunoprecipitated proteins (Figure
17 4).

18 The Ebola virus genes were sequenced from the VEE
19 replicon clones and are listed here as SEQ ID NO:1
20 (GP), 2 (NP), 3 (VP24), 4 (VP30), 5 (VP35), 6 (VP40),
21 and 7 (VP30#2) as described below. The corresponding
22 amino acid sequences of the Ebola proteins expressed
23 from these replicons are listed as SEQ ID NO: 17, 18,
24 19, 20, 21, 22, and 23, respectively. Changes in the
25 DNA sequence relative to the sequence published by
26 Sanchez *et al.* (1993) are described relative to the
27 nucleotide (nt) sequence number from GenBank
28 (accession number L11365).

29 The sequence we obtained for Ebola virus GP (SEQ
30 ID NO:1) differed from the GenBank sequence by a
31 transition from A to G at nt 8023. This resulted in a
32 change in the amino acid sequence from Ile to Val at
33 position 662 (SEQ ID NO: 17).

34 The DNA sequence we obtained for Ebola virus NP
35 (SEQ ID NO:2) differed from the GenBank sequence at
36 the following 4 positions: insertion of a C residue
37 between nt 973 and 974, deletion of a G residue at nt
38 979, transition from C to T at nt 1307, and a

1 transversion from A to C at nt 2745. These changes
2 resulted in a change in the protein sequence from Arg
3 to Glu at position 170 and a change from Leu to Phe at
4 position 280 (SEQ ID NO: 18).

5 The Ebola virus VP24 (SEQ ID NO:3) gene differed
6 from the GenBank sequence at 6 positions, resulting in
7 3 nonconservative changes in the amino acid sequence. -
8 The changes in the DNA sequence of VP24 consisted of a
9 transversion from G to C at nt 10795, a transversion
10 from C to G at nt 10796, a transversion from T to A at
11 nt 10846, a transversion from A to T at nt 10847, a
12 transversion from C to G at nt 11040, and a
13 transversion from C to G at nt 11041. The changes in
14 the amino acid sequence of VP24 consisted of a Cys to
15 Ser change at position 151, a Leu to His change at
16 position 168, and a Pro to Gly change at position 233
17 (SEQ ID NO: 19).

18 We have included 2 different sequences for the
19 Ebola virus VP30 gene (SEQ ID NOS:4 and SEQ ID NO:7).
20 Both of these sequences differ from the GenBank
21 sequence by the insertion of an A residue in the
22 upstream noncoding sequence between nt 8469 and 8470
23 and an insertion of a T residue between nt 9275 and
24 9276 that results in a change in the open reading
25 frame of VP30 and VP30#2 after position 255 (SEQ ID
26 NOS:20 and SEQ ID NO:23). As a result, the C-terminus
27 of the VP30 protein differs significantly from that
28 previously reported. In addition to these 2 changes,
29 the VP30#2 gene in SEQ ID NO:23 contains a
30 conservative transition from T to C at nt 9217.
31 Because the primers originally used to clone the VP30
32 gene into the replicon were designed based on the
33 GenBank sequence, the first clone that we constructed
34 (SEQ ID NO:4) did not contain what we believe to be
35 the authentic C-terminus of the protein. Therefore,
36 in the absence of the VP30 stop codon, the C-terminal
37 codon was replaced with 37 amino acids derived from
38 the vector sequence. The resulting VP30 construct

1 therefore differed from the GenBank sequence in that
2 it contained 32 amino acids of VP30 sequence
3 (positions 256 to 287, SEQ ID NO:20) and 37 amino
4 acids of irrelevant sequence (positions 288 to 324,
5 SEQ ID NO:20) in the place of the C-terminal 5 amino
6 acids reported in GenBank. However, inclusion of 37
7 amino acids of vector sequence in place of the C-
8 terminal amino acid (Pro, SEQ ID NO:23) did not
9 inhibit the ability of the protein to serve as a
10 protective antigen in BALB/c mice. We are currently
11 examining the ability of the new VEE replicon
12 construct (SEQ ID NO:7), which we believe contains the
13 authentic C-terminus of VP30 (VP30#2, SEQ ID NO:23),
14 to protect mice against a lethal Ebola challenge.

15 The DNA sequence for Ebola virus VP35 (SEQ ID
16 NO:5) differed from the GenBank sequence by a
17 transition from T to C at nt 4006, a transition from T
18 to C at nt 4025, and an insertion of a T residue
19 between nt 4102 and 4103. These sequence changes
20 resulted in a change from a Ser to a Pro at position
21 293 and a change from Phe to Ser at position 299 (SEQ
22 ID NO:21). The insertion of the T residue resulted in
23 a change in the open reading frame of VP35 from that
24 previously reported by Sanchez et al. (1993) following
25 amino acid number 324. As a result, Ebola virus VP35
26 encodes for a protein of 340 amino acids, where amino
27 acids 325 to 340 (SEQ ID NO:21) differ from and
28 replace the C-terminal 27 amino acids of the
29 previously published sequence.

30 Sequencing of VP30 and VP35 was also performed
31 on RT/PCR products from RNA derived from cells that
32 were infected with Ebola virus 1976, Ebola virus 1995
33 or the mouse-adapted Ebola virus. The changes noted
34 above for the VRep constructs were also found in these
35 Ebola viruses. Thus, we believe that these changes are
36 real events and not artifacts of cloning.

37 The Ebola virus VP40 differed from the GenBank
38 sequence by a transversion from a C to G at nt 4451

1 and a transition from a G to A at nt 5081. These
2 sequence changes did not alter the protein sequence of
3 VP40 (SEQ ID NO:22) from that of the published
4 sequence.

5 To evaluate the protective efficacy of
6 individual Ebola virus proteins and to determine
7 whether the major histocompatibility (MHC) genes
8 influence the immune response to Ebola virus antigens,
9 two MHC-incompatible strains of mice were vaccinated
10 with VRPs expressing an Ebola protein. As controls for
11 these experiments, some mice were injected with VRPs
12 expressing the nucleoprotein of Lassa virus or were
13 injected with phosphate-buffered saline (PBS).
14 Following Ebola virus challenge, the mice were
15 monitored for morbidity and mortality, and the results
16 are shown in Table 1.

17 The GP, NP, VP24, VP30, and VP40 proteins of
18 Ebola virus generated either full or partial
19 protection in BALB/c mice, and may therefore be
20 beneficial components of a vaccine designed for human
21 use. Vaccination with VRPs encoding the NP protein
22 afforded the best protection. In this case, 100% of
23 the mice were protected after three inoculations and
24 95% of the mice were protected after two inoculations.
25 The VRP encoding VP24 also protected 90% to 95% of
26 BALB/c mice against Ebola virus challenge. In separate
27 experiments (Table 2), two or three inoculations with
28 VRPs encoding the VP24 protein protected BALB/c mice
29 from a high dose (1×10^5 plaque-forming units ($3 \times$
30 10^6 LD₅₀)) of mouse-adapted Ebola virus.

31 Vaccination with VRPs encoding GP protected 52-
32 70% of BALB/c mice. The lack of protection was not
33 due to a failure to respond to the VRP encoding GP, as
34 all mice had detectable Ebola virus-specific serum
35 antibodies after vaccination.

36 Some protective efficacy was also observed in
37 BALB/c mice vaccinated two or three times with VRPs
38 expressing the VP30 protein (55% and 85%,

1 respectively), or the VP40 protein (70% and 80%,
2 respectively). The VP35 protein was not efficacious
3 in the BALB/c mouse model, as only 20% and 26% of the
4 mice were protected after either two or three doses,
5 respectively.

6 Geometric mean titers of viremia were markedly
7 reduced in BALB/c mice vaccinated with VRPs encoding
8 Ebola virus proteins after challenge with Ebola virus,
9 indicating an ability of the induced immune responses
10 to reduce virus replication (Table 1A). In this study,
11 immune responses to the GP protein were able to clear
12 the virus to undetectable levels within 4 days after
13 challenge in some mice.

14 When the same replicons were examined for their
15 ability to protect C57BL/6 mice from a lethal
16 challenge of Ebola virus, only the GP, NP, and VP35
17 proteins were efficacious (Table 1B). The best
18 protection, 95% to 100%, was observed in C57BL/6 mice
19 inoculated with VRPs encoding the GP protein.
20 Vaccination with VRPs expressing NP protected 75% to
21 80% of the mice from lethal disease. In contrast to
22 what was observed in the BALB/c mice, the VP35 protein
23 was the only VP protein able to significantly protect
24 the C57BL/6 mice. In this case, 3 inoculations with
25 VRPs encoding VP35 protected 70% of the mice from
26 Ebola virus challenge. The reason behind the
27 differences in protection in the two mouse strains is
28 not known but is believed to be due to the ability of
29 the immunogens to sufficiently stimulate the cellular
30 immune system. As with the BALB/c mice, the effects
31 of the induced immune responses were also observed in
32 reduced viremias and, occasionally, in a prolonged
33 time to death of C57BL/6 mice.

34 VRPs expressing Ebola virus GP or NP were also
35 evaluated for protective efficacy in a guinea pig
36 model. Sera from vaccinated animals were assayed for
37 antibodies to Ebola by western blotting, IFA, plaque-
38 reduction neutralization, and ELISA. Vaccination with

1 either VRP (GP or NP) induced high titers of
2 antibodies to the Ebola proteins (Table 4) in both
3 guinea pig strains. Neutralizing antibody responses
4 were only detected in animals vaccinated with the VRP
5 expressing GP (Table 4).

6 Vaccination of strain 2 inbred guinea pigs with
7 the GP construct protected 3/5 animals against death
8 from lethal Ebola challenge, and significantly
9 prolonged the mean day of death in one of the two
10 animals that died (Table 4). All of the strain 13
11 guinea pigs vaccinated with the GP construct, alone or
12 in combination with NP, survived lethal Ebola
13 challenge (Table 4). Vaccination with NP alone did not
14 protect either guinea pig strain from challenge with
15 the guinea pig-adapted Ebola virus.

16 To identify the immune mechanisms that mediate
17 protection against Ebola virus and to determine
18 whether antibodies are sufficient to protect against
19 lethal disease, passive transfer studies were
20 performed. One mL of immune sera, obtained from mice
21 previously vaccinated with one of the Ebola virus
22 VRPs, was passively administered to unvaccinated mice
23 24 hours before challenge with a lethal dose of mouse-
24 adapted Ebola virus. Antibodies to GP, but not to NP
25 or the VP proteins, protected mice from an Ebola virus
26 challenge (Table 3). Antibodies to GP protected 75% of
27 the BALB/c mice and 85% of the C57BL/6 mice from
28 death. When the donor sera were examined for their
29 ability to neutralize Ebola virus in a plaque-
30 reduction neutralization assay, a 1:20 to 1:40
31 dilution of the GP-specific antisera reduced the
32 number of viral plaque-forming units by at least 50%
33 (data not shown). In contrast, antisera to the NP and
34 VP proteins did not neutralize Ebola virus at a 1:20
35 or 1:40 dilution. These results are consistent with
36 the finding that GP is the only viral protein found on
37 the surface of Ebola virus, and is likely to induce
38 virus-neutralizing antibodies.

1 Since the NP and VP proteins of Ebola virus are
2 internal virion proteins to which antibodies are not
3 sufficient for protection, it is likely that cytotoxic
4 T lymphocytes (CTLs) are also important for protection
5 against Ebola virus. Initial studies aimed at
6 identifying cellular immune responses to individual
7 Ebola virus proteins expressed from VRPs identified
8 CTL responses to the VP24 and NP proteins (Table 5).
9 One CTL epitope that we identified for the Ebola virus
10 NP is recognized by C57BL/6 (H-2^b) mice, and has an
11 amino acid sequence of, or contained within, the
12 following 11 amino acids: VYQVNNLEEIC (SEQ ID NO:24).
13 Vaccination with EboNPVRP and *in vitro* restimulation
14 of spleen cells with this peptide consistently induces
15 strong CTL responses in C57BL/6 (H-2^b) mice. *In vivo*
16 vaccination to Ebola virus NP is required to detect
17 the CTL activity, as evidenced by the failure of cells
18 from C57BL/6 mice vaccinated with Lassa NP to develop
19 lytic activity to peptide (SEQ ID NO:24) after *in*
20 *vitro* restimulation with it. Specific lysis has been
21 observed using very low effector:target ratios (<2:1).
22 This CTL epitope is H-2^b restricted in that it is not
23 recognized by BALB/c (H-2^d) cells treated the same way
24 (data not shown), and H-2^b effector cells will not
25 lyse MHC-mismatched target cells coated with this
26 peptide.

27 A CTL epitope in the VP24 protein was also
28 identified. It is recognized by BALB/c (H-2^d) mice,
29 and has an amino acid sequence of, or contained
30 within, the following 23 amino acids:
31 LKFINKLDALLVVNYNGLLSSIF (SEQ ID NO:25). In the data
32 shown in Table 5, high (>90%) specific lysis of P815
33 target cells coated with this peptide was observed.
34 The background lysis of cells that were not peptide-
35 coated was also high (>50%), which is probably due to
36 the activity of natural killer cells. We are planning
37 to repeat this experiment using the L5178Y target

1 cells, which are not susceptible to natural killer
2 cells.

3 Future studies will focus on determining the
4 fine specificities of these CTL responses and the
5 essential amino acids that constitute these CTL
6 epitopes. Additional studies to identify other CTL
7 epitopes on Ebola virus GP, NP, VP24, VP30, VP35, and
8 VP40 will be performed. To evaluate the role of these
9 CTLs in protection against Ebola virus, lymphocytes
10 will be restimulated *in vitro* with peptides containing
11 the CTL epitopes, and adoptively transferred into
12 unvaccinated mice prior to Ebola virus challenge. In
13 addition, future studies will examine the CTL
14 responses to the other Ebola virus proteins to better
15 define the roles of the cell mediated immune responses
16 involved in protection against Ebola virus infection.

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2 What is claimed is:

3 1. A DNA fragment which encodes a GP Ebola protein,
4 said DNA fragment comprising the sequence specified in
5 SEQ ID NO:1, or a polynucleotide fragment comprising
6 at least 15 nucleotides.

7

8 2. A DNA fragment which encodes a NP Ebola protein,
9 said DNA fragment comprising the sequence specified in
10 SEQ ID NO:2, or a polynucleotide fragment comprising
11 at least 15 nucleotides.

12

13 3. A DNA fragment which encodes a VP24 Ebola protein,
14 said DNA fragment comprising the sequence specified in
15 SEQ ID NO:3, or a polynucleotide fragment comprising
16 at least 15 nucleotides.

17

18 4. A DNA fragment which encodes a VP30 Ebola protein,
19 said DNA fragment comprising the sequence specified in
20 any of SEQ ID NO:4 and SEQ ID NO:7, or a
21 polynucleotide fragment comprising at least 15
22 nucleotides.

23

24 5. A DNA fragment which encodes a VP35 Ebola protein,
25 said DNA fragment comprising the sequence specified in
26 SEQ ID NO:5, or a polynucleotide fragment comprising
27 at least 15 nucleotides.

28

29 6. A DNA fragment which encodes a VP40 Ebola protein,
30 said DNA fragment comprising the sequence specified in
31 SEQ ID NO:6, or a polynucleotide fragment comprising
32 at least 15 nucleotides.

33

34 7. A DNA fragment which encodes a GP Ebola protein
35 said DNA fragment comprising a DNA sequence encoding
36 at least 5 amino acids specified in SEQ ID NO:17 or a
37 conservative substitution thereof.

1

2 8. A DNA fragment which encodes a NP Ebola protein
3 said DNA fragment comprising a DNA sequence encoding
4 at least 5 amino acids specified in SEQ ID NO:18 or a
5 conservative substitution thereof.

6

7 9. A DNA fragment which encodes a VP24 Ebola protein -
8 said DNA fragment comprising a DNA sequence encoding
9 at least 5 amino acids specified in SEQ ID NO:19 or a
10 conservative substitution thereof.

11

12 10. A DNA fragment which encodes a VP30 Ebola protein
13 said DNA fragment comprising a DNA sequence encoding
14 at least 5 amino acids specified in any of SEQ ID
15 NO:20 and SEQ ID NO:23 or a conservative substitution
16 thereof.

17

18 11. A DNA fragment which encodes a VP35 Ebola protein
19 said DNA fragment comprising a DNA sequence encoding
20 at least 5 amino acids specified in SEQ ID NO:21 or a
21 conservative substitution thereof.

22

23 12. A DNA fragment which encodes a VP40 Ebola protein
24 said DNA fragment comprising a DNA sequence encoding
25 at least 5 amino acids specified in SEQ ID NO:22 or a
26 conservative substitution thereof.

27

28 13. A recombinant DNA construct comprising:
29 (i) a vector, and
30 (ii) at least one of the Ebola virus DNA
31 fragments chosen from the group consisting of SEQ ID
32 NO:1, 2, 3, 4, 5, 6 and 7 or a fragment thereof
33 comprising at least 15 nucleotides.

34

35 14. A recombinant DNA construct comprising:
36 (i) a vector, and
37 (ii) at least one of the Ebola virus DNA
38 fragments chosen from the group consisting of SEQ ID

- 1 NO: 17, 18, 19, 20, 21, 22, 23, 24 and 25 or a
2 conservative substitution thereof.
3
- 4 15. The recombinant DNA construct of claim 13 wherein
5 said DNA fragment induces a cytotoxic T lymphocyte
6 response or antibody response.
7
- 8 16. The recombinant DNA construct of claim 14 wherein
9 said DNA fragment induces a cytotoxic T lymphocyte
10 response or antibody response.
11
- 12 17. A recombinant DNA construct according to claim 13
13 wherein said vector is an expression vector.
14
- 15 18. A recombinant DNA construct according to claim 13
16 wherein said vector is a prokaryotic vector.
17
- 18 19. A recombinant DNA construct according to claim 13
19 wherein said vector is a eukaryotic vector.
20
- 21 20. A recombinant DNA construct according to claim 14
22 wherein said vector is an expression vector.
23
- 24 21. A recombinant DNA construct according to claim 14
25 wherein said vector is a prokaryotic vector.
26
- 27 22. A recombinant DNA construct according to claim 14
28 wherein said vector is a eukaryotic vector.
29
- 30 23. The recombinant DNA construct of claim 17 wherein
31 said vector is a VEE virus replicon vector.
32
- 33 24. The recombinant DNA construct of claim 20 wherein
34 said vector is a VEE virus replicon vector.
35
- 36 25. The recombinant DNA construct according to claim
37 23 wherein said Ebola virus DNA fragments are from
38 Ebola Zaire 1976.

1

2 26. The recombinant DNA construct according to claim
3 25 wherein said construct is VRepEboVP24.

4

5 27. The recombinant DNA construct according to claim
6 25 wherein said construct is VRepEboVP30.

7

8 28. The recombinant DNA construct according to claim
9 25 wherein said construct is VRepEboVP35.

10

11 29. The recombinant DNA construct according to claim
12 25 wherein said construct is VRepEboVP40.

13

14 30. The recombinant DNA construct according to claim
15 25 wherein said construct is for VRepEboNP.

16

17 31. The recombinant DNA construct according to claim
18 25 wherein said construct is for VRepEboGP.

19

20 32. The recombinant DNA construct according to claim
21 25 wherein said construct is for VRepEboVP30(#2).

22

23 33. Self replicating RNA produced from a construct
24 chosen from the group consisting of EboVP24ReP,
25 EboVP30ReP, EboVP35ReP, EboVP40ReP, EboVPNPReP,
26 EboVPGPreP, and EboVP30ReP(#2).

27

28 34. Infectious alphavirus particles produced from
29 packaging the self replicating RNA of claim 33.

30

31 35. A pharmaceutical composition comprising infectious
32 alphavirus particles according to claim 34 in an
33 effective immunogenic amount in a pharmaceutically
34 acceptable carrier and/or adjuvant.

35

36 36. A host cell transformed with a recombinant DNA
37 construct according to claim 13.

1

2 37. A host cell transformed with a recombinant DNA
3 construct according to claim 14.

4

5 38. A host cell according to claim 36 wherein said
6 host cell is prokaryotic.

7

8 39. A host cell according to claim 36 wherein said
9 host cell is eukaryotic.

10

11 40. A host cell according to claim 37 wherein said
12 host cell is prokaryotic.

13

14 41. A host cell according to claim 37 wherein said
15 host cell is eukaryotic.

16

17 42. A method for producing Ebola virus proteins
18 comprising culturing the cells according to claim 36
19 under conditions such that said DNA fragment is
20 expressed and said Ebola protein is produced.

21

22 43. A method for producing Ebola virus proteins
23 comprising culturing the cells according to claim 37
24 under conditions such that said DNA fragment is
25 expressed and said Ebola protein is produced.

26

27 44. A method for producing Ebola virus proteins
28 comprising culturing the cells according to claim 38
29 under conditions such that said DNA fragment is
30 expressed and said Ebola protein is produced.

31

32 45. A method for producing Ebola virus proteins
33 comprising culturing the cells according to claim 39
34 under conditions such that said DNA fragment is
35 expressed and said Ebola protein is produced.

36

37

- 1 46. An isolated and purified Ebola GP protein
2 specified in SEQ ID NO:17 and conservative
3 substitutions thereof, or an immunologically
4 identifiable portion thereof.
5
- 6 47. An isolated and purified Ebola NP protein
7 specified in SEQ ID NO:18 and conservative
8 substitutions thereof or an immunologically
9 identifiable portion thereof.
10
- 11 48. An isolated and purified Ebola VP24 protein
12 specified in SEQ ID NO:19 and conservative
13 substitutions thereof or an immunologically
14 identifiable portion thereof.
15
- 16 49. An isolated and purified Ebola VP30 protein
17 specified in any of SEQ ID NO:20 and SEQ ID NO:23 and
18 conservative substitutions thereof or an
19 immunologically identifiable portion thereof.
20
- 21 50. An isolated and purified Ebola VP35 protein
22 specified in SEQ ID NO:21 and conservative
23 substitutions thereof or an immunologically
24 identifiable portion thereof.
25
- 26 51. An isolated and purified Ebola VP40 protein
27 specified in SEQ ID NO:22 and conservative
28 substitutions thereof or an immunologically
29 identifiable portion thereof.
30
- 31 52. An antibody to a peptide encoded by the sequence
32 specified in SEQ ID NO:17, 18, 19, 20, 21, 22, 23, 24,
33 and 25.
34
- 35 53. A method for detecting Ebola virus infection
36 comprising contacting a sample from a subject
37 suspected of having Ebola virus infection with a
38 antibody according to claim 52 and detecting the

1 presence or absence by detecting the presence or
2 absence of a complex formed between the Ebola protein
3 and antibodies specific therefor.
4

5 54. A method for detecting the presence or absence of
6 Ebola virus GP RNA in a sample using the polymerase
7 chain reaction using primers for Ebola GP nucleic acid
8 sequence specified in SEQ ID NO:1 for GP.
9

10 55. An Ebola infection diagnostic kit comprising at
11 least 12 consecutive nucleotides of SEQ ID NO:1
12 specific for the amplification of DNA or RNA of Ebola
13 virus in a sample using the polymerase chain reaction
14 and ancillary reagents suitable for use in such a
15 reaction for detecting the presence or absence of
16 Ebola virus DNA or RNA in a sample.
17

18 56. A vaccine for Ebola comprising alphavirus
19 particles of claim 34.
20

21 57. A method for the diagnosis of Ebola virus
22 infection comprising the steps of:
23 (i) contacting a sample from an individual
24 suspected of having Ebola virus infection with an
25 antibody to Ebola proteins according to claim 52; and
26 (ii) detecting the presence or absence of Ebola
27 virus infection by detecting the presence or absence
28 of a complex formed between Ebola proteins and
29 antibodies specific therefor.
30

31 58. A pharmaceutical composition comprising the self
32 replicating RNA of claim 33 in an effective immunogenic
33 amount in a pharmaceutically acceptable carrier and/or
34 adjuvant.
35

36 59. A pharmaceutical composition comprising one or more
37 recombinant DNA constructs chosen from the group
38 consisting of VRepEboVP24, VRepEboVP30, VRepEboVP35,

1 VRepEboVP40, VRepEboNP, VRepEboGP, and VRepEboVP30(#2),
2 in a pharmaceutically acceptable amount, in a
3 pharmaceutically acceptable carrier and/or adjuvant.
4

5 60. A pharmaceutical composition comprising comprising a
6 peptide encoded by any of SEQ ID NO:24 and SEQ ID NO:25,
7 in a pharmaceutically acceptable amount, in a
8 pharmaceutically acceptable carrier and/or adjuvant.
9

10

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31

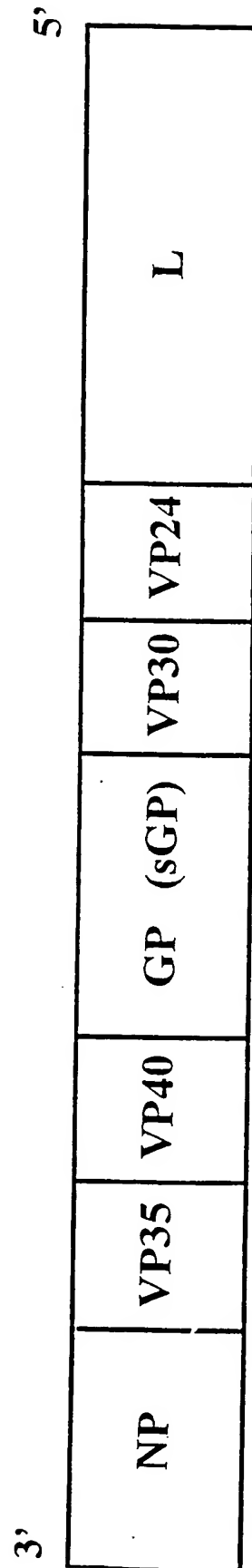
32

33

34

FIGURE 1

Organization of the Ebola Virus Genome



- NP Major Nucleocapsid Protein
- VP35 Phosphoprotein
- VP40 Membrane-Associated Matrix Protein
- GP Transmembrane Glycoprotein
- sGP Secreted Glycoprotein
- VP30 Ribonucleoprotein Associated (Minor)
- VP24 Membrane-Associated Protein (Minor)
- L RNA-Dependent RNA Polymerase

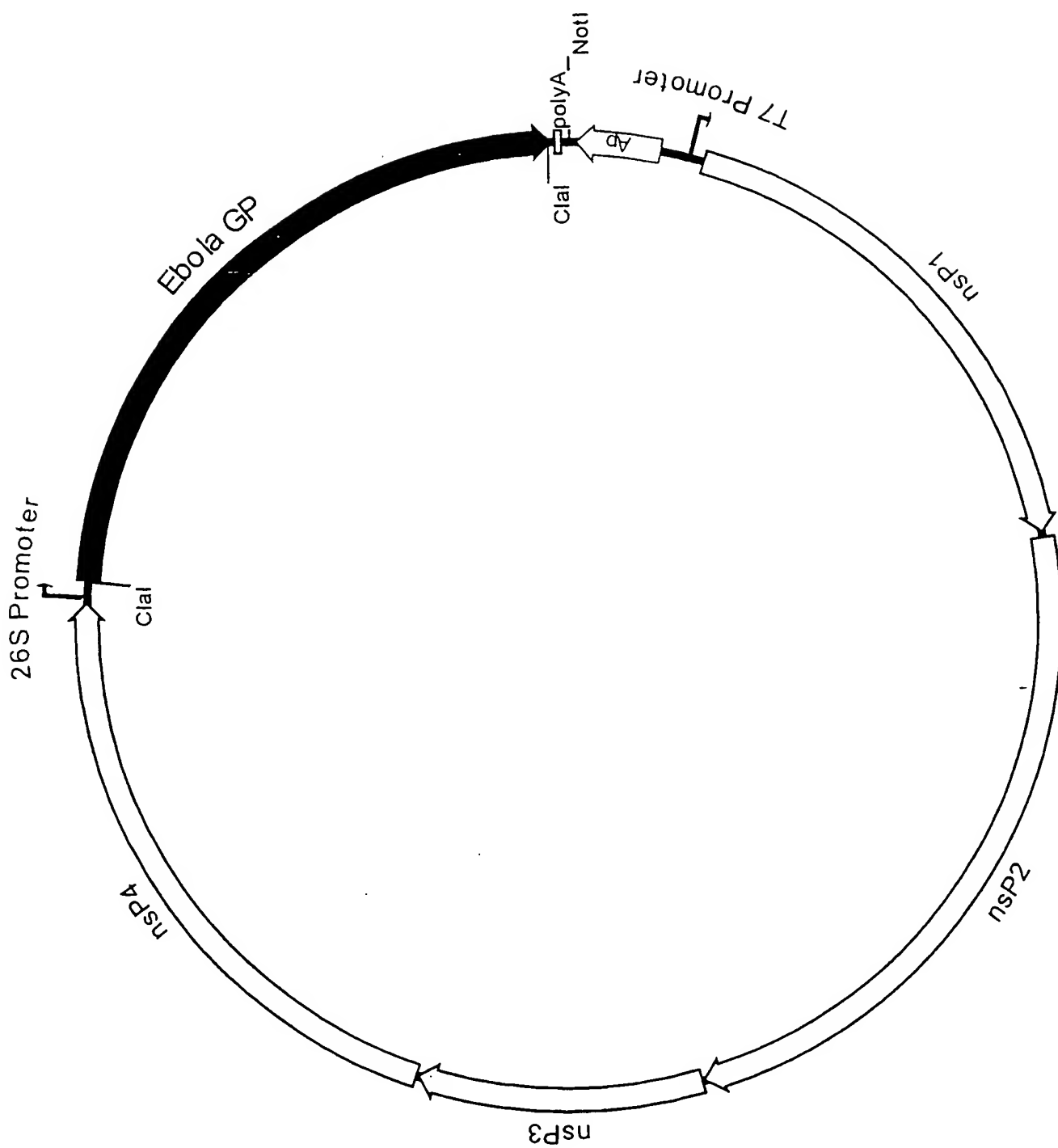
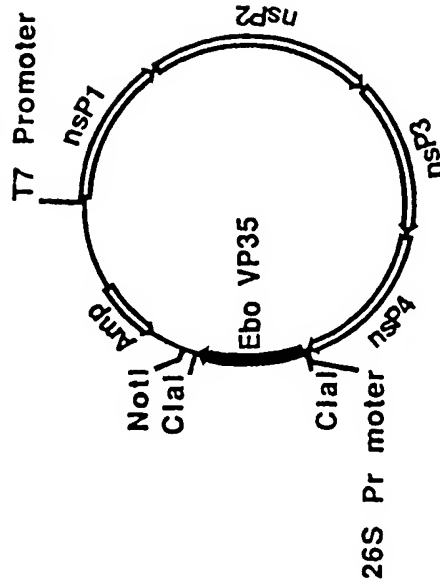
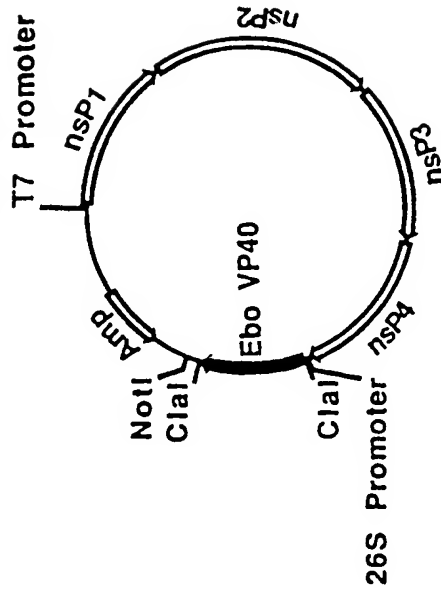


FIGURE 2B

Ebola VP35 Replicon



Ebola VP40 Replicon



Ebola VP30 Replicon (#2)

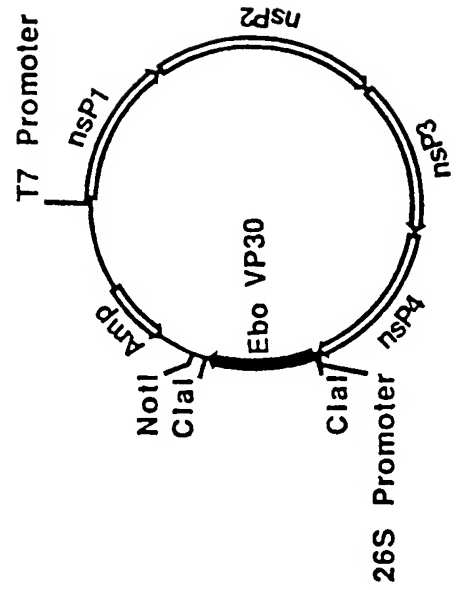


FIGURE 2C

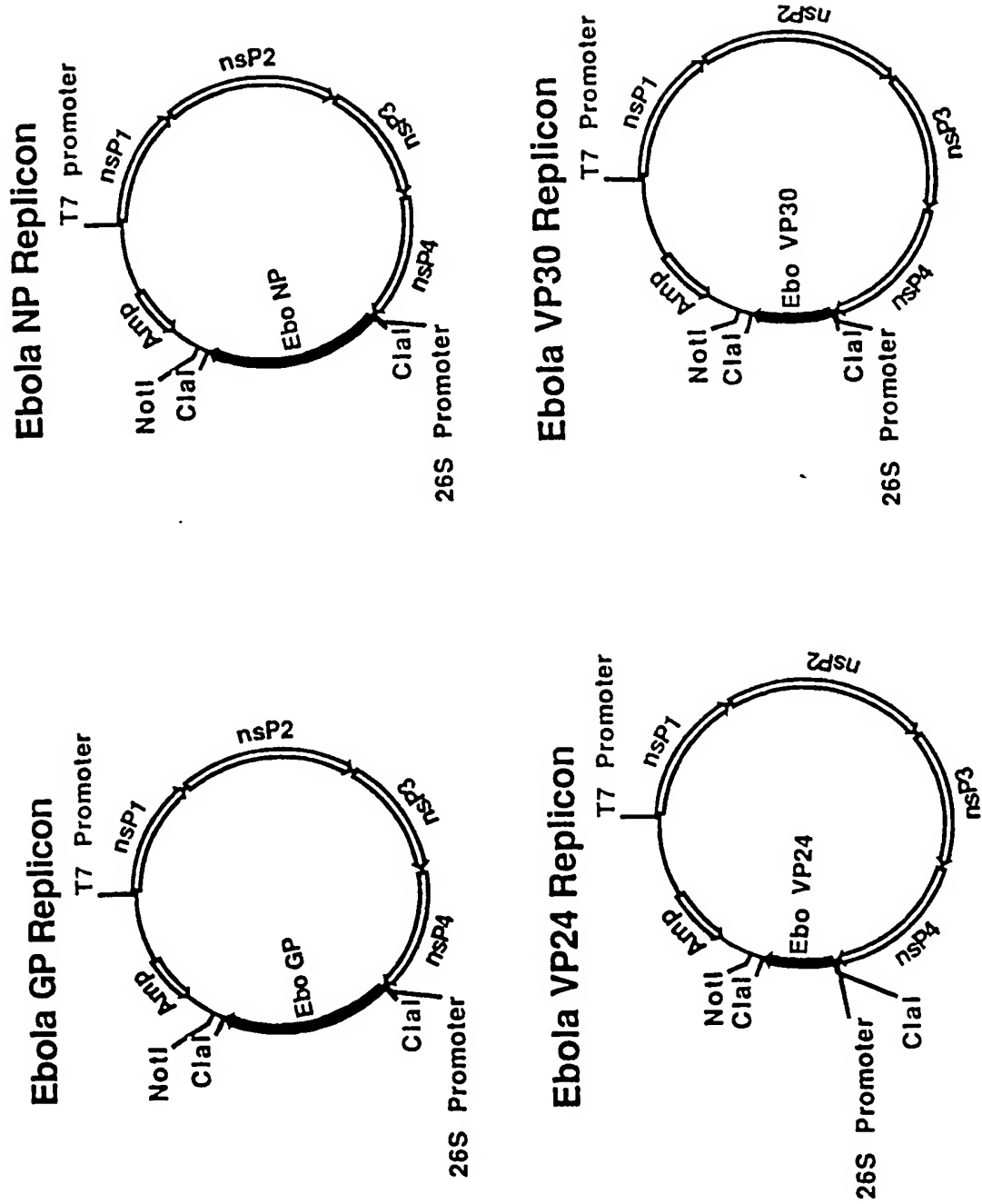


FIGURE 2

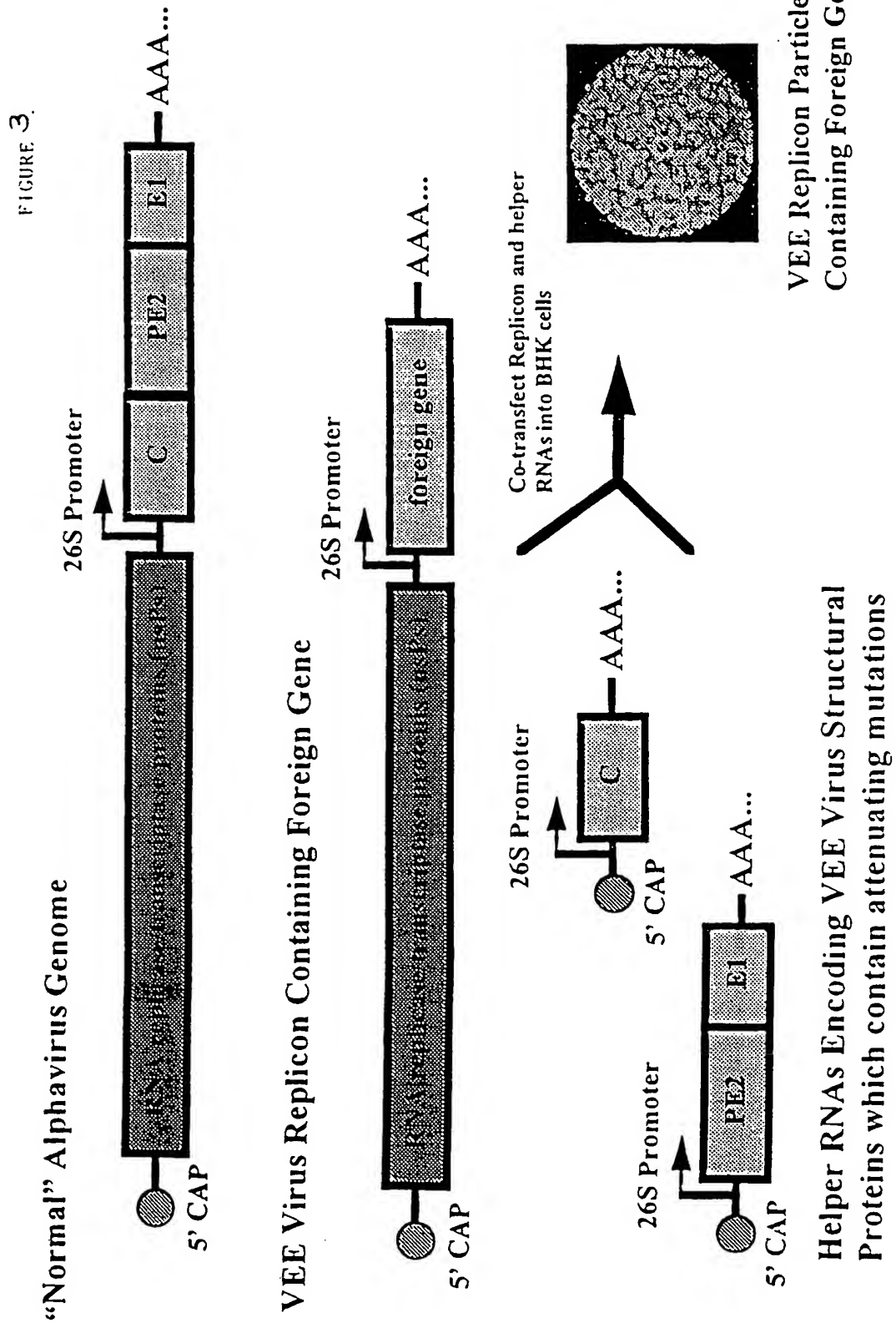
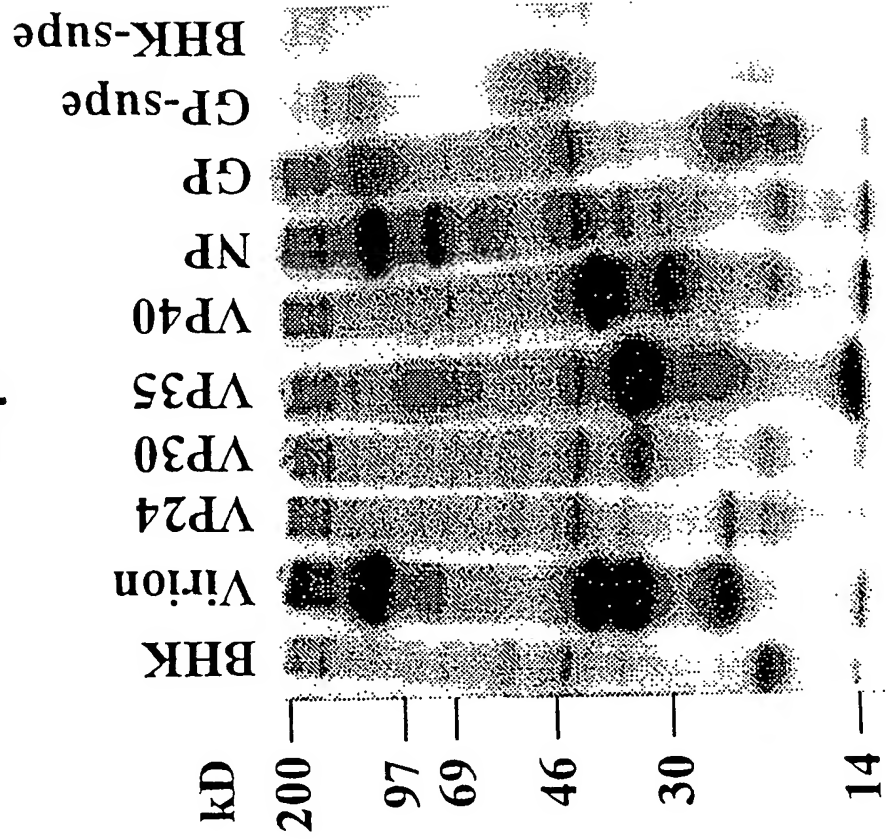


FIGURE 4

Ebola Proteins Expressed from VEE Replicons



Sequence ID NO: 1 (Ebola GP DNA sequence in replicon):

```

ATCGATAAGC TCGGAATTCG AGCTCGCCCG GGGATCCTCT AGAGTCGACA ACAACACAAT
GGGCGTTACA GGAATATTGC AGTTACCTCG TGATCGATTTC AAGAGGACAT CATTCTTTCT
TTGGGTAATT ATCCTTTTCC AAAGAACATT TTCCATCCCA CTTGGAGTCA TCCACAATAG
CACATTACAG GTTAGTGATG TCGACAAACT AGTTTGTCGT GACAAACTGT CATCCACAAA
TCAATTGAGA TCAGTTGGAC TGAATCTCGA AGGGAATGGA GTGGCAACTG ACGTGCCATC
TGCAACTAAA AGATGGGGCT TCAGGTCCGG TGTCCCACCA AAGGTGGTCA ATTATGAAAGC
TGGTGAATGG GCTGAAAAC TCTACAATCT TGAAATCAAA AAACCTGACG GGAGTGAGTG
TCTACCAGCA GCGCCAGACG GGATTCGGGG CTTCCCCCGG TGCCGGTATG TGCACAAAGT
ATCAGGAACG GGACCGTGTG CCGGAGACTT TGCCTTCCAT AAAGAGGGTG CTTTCTTCCT
GTATGATCGA CTTGCTTCCA CAGTTATCTA CCGAGGAACG ACTTTCGCTG AAGGTGTCGT
TGCATTTCTG ATACTGCCCC AAGCTAAGAA GGACTTCTTC AGCTCACACC CCTTGAGAGA
GCCGGTCAAT GCAACGGAGG ACCCGTCTAG TGGCTACTAT TCTACCACAA TTAGATATCA
GGCTACCGGT TTTGGAACCA ATGAGACAGA GTACTTGTTT GAGGTTGACA ATTTGACCTA
CGTCCAACCT GAATCAAGAT TCACACCACA GTTTCTGCTC CAGCTGAATG AGACAATATA
TACAAGTGGG AAAAGGAGCA ATACCACGGG AAAACTAATT TGGAAGGTCA ACCCCGAAAT
TGATACAACA ATCGGGGAGT GGGCCTTCTG GGAAACTAAA AAAACCTCA CTAGAAAAAT
TCGCAGTGAA GAGTTGTCTT TCACAGTTGT ATCAAACGGA GCCAAAAACA TCAGTGGTCA
GAGTCCGGCG CGAACTTCTT CCGACCCAGG GACCAACACA ACAACTGAAG ACCACAAAAT
CATGGCTTCA GAAAATTCTT CTGCAATGGT TCAAGTGCAC AGTCAAGGAA GGGGAAGCTGC
AGTGTGCGAT CTAACAACCC TTGCCACAAT CTCCACGAGT CCCCATCCC TCACAACCAA
ACCAGGTCGG GACAACAGCA CCCATAATAC ACCCGTGTAT AAACCTGACA TCTCTGAGGC
AACTCAAGTT GAACAACATC ACCGCAGAAC AGACAACGAC AGCACAGCCT CCGACACTCC
CTCTGCCACG ACCGCAGCCG GACCCCCAAA AGCAGAGAAC ACCAACACGA GCAAGAGCAC
TGACTTCCTG GACCCCGCCA CCACAACAAG TCCCCAAAAC CACAGCGAGA CCGCTGGCAA
CAACAACACT CATCACCAG ATACCGGAGA AGAGAGTGCC AGCAGCGGGA AGCTAGGCTT
AATTACCAAT ACTATTGCTG GAGTCGCAGG ACTGATCACA GGCGGGAGAA GAACTCGAAG
AGAAGCAATT GTCAATGCTC AACCCTAATG CAACCCTAAT TTACATTACT GGACTACTCA
GGATGAAGGT GCTGCAATCG GACTGGCCTG GATACCATAT TTCGGGCCAG CAGCCGAGGG
AATTTACATA GAGGGGCTAA TGCACAATCA AGATGGTTTA ATCTGTGGGT TGAGACAGCT
GGCCAACGAG ACGACTCAAG CTCTTCAACT GTTCTTGAGA GCCACAAC TG AGCTACGCAC
CTTTTCAATC CTCAACCGTA AGGCAATTGA TTTCTTGCTG CAGCGATGGG GCGGCACATG
CCACATTCTG GGACCGGACT GCTGTATCGA ACCACATGAT TGGACCAAGA ACATAACAGA
CAAAATTGAT CAGATTATTC ATGATTTTGT TGATAAAACC CTTCCGGACC AGGGGGACAA
TGACAATTGG TGGACAGGAT GGAGACAATG GATACCGGCA GGTATTGGAG TTACAGGCGT
TGTAATTGCA GTTATCGCTT TATTCTGTAT ATGCAAATTT GTCTTTTAGT TTTTCTTCAG
ATTGCTTCAT GGAAAAGCTC AGCCTCAAAT CAATGAAACC AGGATTTAAT TATATGGATT
ACTTGAATCT AAGATTACTT GACAAATGAT AATATAATAC ACTGGAGCTT TAAACATAGC
CAATGTGATT CTAACCTCTT TAAACTCACA GTTAATCATA AACAAGGTTT GAGTCGACCT
GCAGCCAAGC TTATCGAT

```

Sequence ID NO: 2 (Ebola NP DNA sequence in replicon):

```

ATCGATAAGC TTGGCTGCAG GTCGACTCTA GAGGATCCGA GTATGGATTTC TCGTCCTCAG
AAAATCTGGA TGGCGCCGAG TCTCACTGAA TCTGACATGG ATTACCACAA GATCTTGACA
GCAGGTCTGT CCGTTCAACA GGGGATTGTT CGGCAAAGAG TCATCCCAGT GTATCAAGTA
AACAATCTTG AAGAAATTTG CCAACTTATC ATACAGGCCT TTGAAGCAGG TGTTGATTTT
CAAGAGAGTG CGGACAGTTT CCTTCTCATG CTTTGTCTTC ATCATGCGTA CCAGGGAGAT
TACAAACTTT TCTTGGAAG TGCGCAGTC AAGTATTTGG AAGGGCACGG GTTCCGTTTT
GAAGTCAAGA AGCGTGATGG AGTGAAGCGC CTTGAGGAAT TGCTGCCAGC AGTATCTAGT
GGAAAAACA TTAAGAGAAC ACTTGCTGCC ATGCCGGAAG AGGAGACAAC TGAAGCTAAT
GCCGGTCAGT TTCTCTCCTT TGCAAGTCTA TTCCTTCCGA AATTGGTAGT AGGAGAAAAG
GCTTGCCCTG AGAAGGTTCA AAGGCAAATT CAAGTACATG CAGAGCAAGG ACTGATACAA
TATCCAACAG CTTGGCAATC AGTAGGACAC ATGATGGTGA TTTTCCGTTT GATGCGAACA
AATTTTCTGA TCAAATTTCT CCTAATACAC CAAGGGATGC ACATGGTTGC CGGGCATGAT
GCCAACGATG CTGTGATTTT AAATTCAGTG GCTCAAGCTC GTTTTTCAGG CTTATTGATT
GTCAAAACAG TACTTGATCA TATCCTACAA AAGACAGAAC GAGGAGTTCTG TCTCCATCCT
CTTGCAAGGA CCGCCAAGGT AAAAAATGAG GTGAACCTCT TTAAGGCTGC ACTCAGCTCC
CTGGCCAAGC ATGGAGAGTA TGCTCCTTTC GCCCGACTTT TGAACCTTTC TGGAGTAAAT
AATCTTGAGC ATGGTCTTTT CCCTCAACTA TCGGCAATTG CACTCGGAGT CGCCACAGCA
CACGGGAGTA CCCTCGCAGG AGTAAATGTT GGAGAACAGT ATCAACAAC T CAGAGAGGCT
GCCACTGAGG CTGAGAAGCA ACTCCAACAA TATGCAGAGT CTCGCGAACT TGACCATCTT
GGACTTGATG ATCAGGAAAA GAAATTTCTT ATGAACCTCC ATCAGAAAAA GAACGAAATC
AGCTTCCAGC AAACAAACGC TATGGTAACT CTAAGAAAAG AGCGCCTGGC CAAGCTGACA
GAAGCTATCA CTGCTGCGTC ACTGCCAAA ACAAGTGGAC ATTACGATGA TGATGACGAC
ATTCCCTTTC CAGGACCCAT CAATGATGAC GACAATCCTG GCCATCAAGA TGATGATCCG
ACTGACTCAC AGGATACGAC CATTCCCGAT GTGGTGGTTG ATCCCGATGA TGGAAAGCTAC
GGCGAATACC AGAGTTACTC GGAAAACGGC ATGAATGCAC CAGATGACTT GGTCTTATTC
GATCTAGACG AGGACGACGA GGACACTAAG CCAGTGCCTA ATAGATCGAC CAAGGGTGGA
CAACAGAAGA ACAGTCAAAA GGGCCAGCAT ATAGAGGGCA GACAGACACA ATCCAGGCCA
ATTCAAAATG TCCCAGGCC TCACAGAACA ATCCACCACG CCAGTGCGCC ACTCACGGAC
AATGACAGAA GAAATGAACC CTCCGGCTCA ACCAGCCCTC GCATGCTGAC ACCAATTAAC
GAAGAGGCAG ACCCACTGGA CGATGCCGAC GACGAGACGT CTAGCCTTCC GCCCTTGAGG
TCAGATGATG AAGAGCAGGA CAGGGACGGA ACTTCCAACC GCACACCCAC TGTCGCCCCA
CCGGCTCCCG TATACAGAGA TCACTCTGAA AAGAAAGAAC TCCCGCAAGA CGAGCAACAA
GATCAGGACC ACACTCAAGA GGCCAGGAAC CAGGACAGTG ACAACACCCA GTCAGAACAC
TCTTTTGAGG AGATGTATCG CCACATTCTA AGATCACAGG GGCCATTTGA TGCTGTTTTG
TATTATCATA TGATGAAGGA TGAGCCTGTA GTTTTCAGTA CCAGTGATGG CAAAGAGTAC
ACGTATCCAG ACTCCCTTGA AGAGGAATAT CCACCATGGC TCACTGAAAA AGAGGCTATG
AATGAAGAGA ATAGATTTGT TACATTGGAT GGTCAACAAT TTTATTGGCC GGTGATGAAT
CACAAGAATA AATTCATGGC AATCCTGCAA CATCATCAGT GAATGAGCAT GGAACAATGG
GATGATTCAA CCGACAAATA GCTAACATTA AGTAGTCCAG GAACGAAAAC AGGAAGAATT
TTTGATGTCT AAGGTGTGAA TTATTATCAC AATAAAAGTG ATTCTTATTT TTGAATTTGG
GCGAGCTCGA ATTCCCGAGC TTATCGAT

```

Sequence ID NO: 3 (Ebola VP24 DNA sequence in replicon):

```

ATCGATCTCC AGACACCAAG CAAGACCTGA GAAAAAACCA TGGCTAAAGC TACGGGACGA
TACAATCTAA TATCGCCCAA AAAGGACCTG GAGAAAGGGG TTGTCTTAAG CGACCTCTGT
AACTTCTTAG TTAGCCAAAC TATTCAGGGG TGGAAAGGTTT ATTGGGCTGG TATTGAGTTT
GATGTGACTC ACAAAGGAAT GGCCCTATTG CATAGACTGA AAACATAATGA CTTTGCCCCCT
GCATGGTCAA TGACAAGGAA TCTCTTTCCCT CATTTATTTT AAAATCCGAA TTCCACAATT
GAATCACCGC TGTGGGCATT GAGAGTCATC CTTGCAGCAG GGATACAGGA CCAGCTGATT
GACCAGTCTT TGATTGAACC CTTAGCAGGA GCCCTTGGTC TGATCTCTGA TTGGCTGCTA
ACAACCAACA CTAACCATTT CAACATGCGA ACACAACGTG TCAAGGAACA ATTGAGCCTA
AAAATGCTGT CGTTGATTCG ATCCAATATT CTCAAGTTTA TTAACAAATT GGATGCTCTA
CATGTCGTGA ACTACAACGG ATTGTTGAGC AGTATTGAAA TTGGAACCTCA AAATCATACA
ATCATCATAA CTCGAACCTAA CATGGGTTTT CTGGTGGAGC TCCAAGAACC CGACAAATCG
GCAATGAACC GCATGAAGCC TGGGCCGGCG AAATTTTCCC TCCTTCATGA GTCCACACTG
AAAGCATTTA CACAAGGATC CTCGACACGA ATGCAAAGTT TGATTCTTGA ATTTAATAGC
TCTCTTGCTA TCTAACTAAG GTAGAATACT TCATATTGAG CTAACCTATA TATGCTGACT
CATCGAT

```

Sequence ID NO: 4 (Ebola VP30 DNA sequence in replicon):

```

ATCGATCAGA TCTGCGAACC GGTAGAGTTT AGTTGCAACC TAACACACAT AAAGCATTGG
TCAAAAAGTC AATAGAAATT TAAACAGTGA GTGGAGACAA CTTTTAAATG GAAGCTTCAT
ATGAGAGAGG ACGCCACGA GCTGCCAGAC AGCATTCAAG GGATGGACAC GACCACCATG
TTCGAGCAGC ATCATCATCC AGAGAGAATT ATCGAGGTGA GTACCGTCAA TCAAGGAGCG
CCTCACAAGT GCGCGTTCCT ACTGTATTTT ATAAGAAGAG AGTTGAACCA TTAACAGTTC
CTCCAGCACC TAAAGACATA TGTCCGACCT TGA AAAAAGG ATTTTGTGT GACAGTAGTT
TTTGCAAAAA AGATCACCAG TTGGAGAGTT TAACTGATAG GGAATTACTC CTACTAATCG
CCCGTAAGAC TTGTGGATCA GTAGAACAAC AATTAAATAT AACTGCACCC AAGGACTCGC
GCTTAGCAAA TCCAACGGCT GATGATTTCC AGCAAGAGGA AGGTCCAAA ATTACCTTGT
TGACACTGAT CAAGACGGCA GAACACTGGG CGAGACAAGA CATCAGAACC ATAGAGGATT
CAAAATTAAG AGCATTGTTG ACTCTATGTG CTGTGATGAC GAGGAAATTC TCAAAATCCC
AGCTGAGTCT TTTATGTGAG ACACACCTAA GCGCGAGGG GCTTGGGCAA GATCAGGCAG
AACCCGTTCT CGAAGTATAT CAACGATTAC ACAGTGATAA AGGAGGCAGT TTTGAAGCTG
CACTATGGCA ACAATGGGAC CTACAATCCC TAATTATGTT TATCACTGCA TTCTTGAATA
TTGCTCTCCA GTTACCGTGT GAAAGTTCTG CTGTCTGTGT TTCAGGGTTA AGAACATTGG
TTCCTCAATC AGATAATGAG GAAGCTTCAA CCAACCCGGG GACATGCTCA TGGTCTGATG
AGGTACATC GAT

```

Sequence ID NO: 5 (Ebola VP35 DNA sequence in replicon):

```

ATCGATAGAA AAGCTGGTCT AACAAAGATGA CAACTAGAAC AAAGGGCAGG GGCCATACTG
CGGCCACGAC TCAAAACGAC AGAATGCCAG GCCCTGAGCT TTCGGGCTGG ATCTCTGAGC
AGCTAATGAC CGGAAGAATT CCTGTAAGCG ACATCTTCTG TGATATTGAG AACAAATCCAG
GATTATGCTA CGCATCCCAA ATGCAACAAA CGAAGCCAAA CCCGAAGACG CGCAACAGTC
AAACCCAAAC GGACCCAATT TGCAATCATA GTTTTGAGGA GGTAGTACAA ACATTGGCTT
CATTGGCTAC TGTTGTGCAA CAACAAACCA TCGCATCAGA ATCATTAGAA CAACGCATTA
CGAGTCTTGA GAATGGTCTA AAGCCAGTTT ATGATATGGC AAAAACAATC TCCTCATTGA
ACAGGGTTTG TGCTGAGATG GTTGCAAAAT ATGATCTTCT GGTGATGACA ACCGGTCGGG
CAACAGCAAC CGCTGCGGCA ACTGAGGCTT ATTGGGCCGA ACATGGTCAA CCACCACCTG
GACCATCACT TTATGAAGAA AGTGCGATTC GGGGTAAGAT TGAATCTAGA GATGAGACCG
TCCCTCAAAG TGTTAGGGAG GCATTCAACA ATCTAAACAG TACCACTTCA CTAAGTGAAG
AAAATTTTGG GAAACCTGAC ATTTTCGGCA AGGATTTGAG AAACATTATG TATGATCACT
TGCCTGGTTT TGGAAGTGCT TTCCACCAAT TAGTACAAGT GATTTGTAAA TTGGGAAAAG
ATAGCAACTC ATTGGACATC ATTCATGCTG AGTTCCAGGC CAGCCTGGCT GAAGGAGACT
CTCCTCAATG TGCCCTAATT CAAATTACAA AAAGAGTTCC AATCTTCCAA GATGCTGCTC
CACCTGTCAT CCACATCCGC TCTCGAGGTG ACATTCCCCG AGCTTGCCAG AAAAGCTTGC
GTCCAGTCCC ACCATCGCCC AAGATTGATC GAGGTGCGGT ATGTGTTTTT CAGCTTCAAG
ATGGTAAAAC ACTTGACTC AAAATTTGAG CCAATCTCCC TTCCCTCCGA AAGAGGCGAA
TAATAGCAGA GGCTTCAACT GCTGAACTAT AGGGTACGTT ACATTAATGA TACACTTGTC
AGATCGAT

```

Sequence ID NO: 6 (Ebola VP40 DNA sequence in replicon):

```

ATCGATCCTA CCTCGGCTGA GAGAGTGTTT TTTCATTAAC CTTTCATCTTG TAAACGTTGA
GCAAAATTGT TAAAAATATG AGGCGGGTTA TATTGCCTAC TGCTCCTCCT GAATATATGG
AGGCCATATA CCCTGTCAGG TCAAATTCAA CAATTGCTAG AGGTGGCAAC AGCAATACAG
GCTTCCTGAC ACCGGAGTCA GTCAATGGGG ACACTCCATC GAATCCACTC AGGCCAATTG
CCGATGACAC CATCGACCAT GCCAGCCACA CACCAGGCAG TGTGTCATCA GCATTCATCC
TTGAAGCTAT GGTGAATGTC ATATCGGGCC CCAAAGTGCT AATGAAGCAA ATTCCAATTT
GGCTTCCTCT AGGTGTCGCT GATCAAAAAGA CCTACAGCTT TGACTCAACT ACGGCCGCCA
TCATGCTTGC TTCATACACT ATCACCATT TCGGCAAGGC AACCAATCCA CTTGTCAGAG
TCAATCGGCT GGTTCCTGGA ATCCCGGATC ATCCCTCAG GCTCCTGCGA ATTGGAAACC
AGGCTTTCCT CCAGGAGTTC GTTCTTCCGC CAGTCCAACT ACCCCAGTAT TTCACCTTTG
ATTTGACAGC ACTCAAACCTG ATCACCACAC CACTGCCTGC TGCAACATGG ACCGATGACA
CTCCAACAGG ATCAAATGGA GCGTTGCGTC CAGGAATTTT ATTTTCATCCA AAACCTCGCC
CCATTCTTTT ACCCAACAAA AGTGGGAAGA AGGGGAACAG TGCCGATCTA ACATCTCCGG
AGAAAATCCA AGCAATAATG ACTTCACTCC AGGACTTTAA GATCGTTCCA ATTGATCCAA
CCAAAAATAT CATGGGAATC GAAGTGCCAG AAACCTCTGGT CCACAAGCTG ACCGGTAAGA
AGGTGACTTC TAAAAATGGA CAACCAATCA TCCCTGTTCT TTTGCCAAAG TACATTGGGT
TGGACCCGGT GGCTCCAGGA GACCTCACCA TGGTAATCAC ACAGGATTGT GACACGTGTC
ATTCTCCTGC AAGTCTTCCA GCTGTGATTG AGAAGTAATT GCAATAATTG ACTCAGATCC
AGTTTTATAG AATCTTCTCA GGGATAGTGC ATAACATATC GAT

```

Sequence ID NO: 7 (^{#2}new Ebola VP30 DNA sequence in replicon):

ATCGATCAGA	TCTGCGAACC	GGTAGAGTTT	AGTTGCAACC	TAACACACAT	AAAGCATTGG
TCAAAAAGTC	AATAGAAATT	TAAACAGTGA	GTGGAGACAA	CTTTTAAATG	GAAGCTTCAT
ATGAGAGAGG	ACGCCCACGA	GCTGCCAGAC	AGCATTCAAG	GGATGGACAC	GACCACCATG
TTCGAGCACG	ATCATCATCC	AGAGAGAATT	ATCGAGGTGA	GTACCGTCAA	TCAAGGAGCG
CCTCACAAGT	GCGCGTTCCT	ACTGTATTTT	ATAAGAAGAG	AGTTGAACCA	TTAACAGTTC
CTCCAGCACC	TAAAGACATA	TGTCCGACCT	TGAAAAAAGG	ATTTTTGTGT	GACAGTAGTT
TTTGCAAAAA	AGATCACCAG	TTGGAGAGTT	TAACTGATAG	GGAATTACTC	CTACTAATCG
CCCGTAAGAC	TTGTGGATCA	GTAGAACAAC	AATTAAATAT	AACTGCACCC	AAGGACTCGC
GCTTAGCAAA	TCCAACGGCT	GATGATTTCC	AGCAAGAGGA	AGGTCCAAAA	ATTACCTTGT
TGACACTGAT	CAAGACGGCA	GAACACTGGG	CGAGACAAGA	CATCAGAACC	ATAGAGGATT
CAAAATTAAG	AGCATTGTTG	ACTCTATGTG	CTGTGATGAC	GAGGAAATTC	TCAAAATCCC
AGCTGAGTCT	TTTATGTGAG	ACACACCTAA	GGCGCGAGGG	GCTTGGGCAA	GATCAGGCAG
AACCCGTTCT	CGAAGTATAT	CAACGATTAC	ACAGTGATAA	AGGAGGCAGT	TTTGAAGCTG
CACTATGGCA	ACAATGGGAC	CGACAATCCC	TAATCATGTT	TATCACTGCA	TTCTTGAATA
TTGCTCTCCA	GTTACCGTGT	GAAAGTTCTG	CTGTCGTTGT	TTCAGGGTTA	AGAACATTGG
TTCTTCAATC	AGATAATGAG	GAAGCTTCAA	CCAACCCGGG	GACATGCTCA	TGGTCTGATG
AGGGTACCCC	TTAATAAGGC	TGACTAAAAC	ACTATATAAC	CTTCTACTTG	ATCACAATAC
TCCGTATACC	TATCATCATA	TATTTAATCA	AGACGATATC	CTTTAAACT	TATTCAGTAC
TATAATCACT	CTCGTTTCAA	ATTAATAAGA	TGTGCATGAT	TGCCCTAATA	TATGAAGAGG
TATGATACAA	CCCTAACAGA	TCGAT			

Sequence ID NO: 8 (Ebola VP24 forward primer):

5'-GGGATCGATCTCCAGACACCAAGCAAGACC-3'

Sequence ID NO: 9 (Ebola VP24 reverse primer):

5'-GGGATCGATGAGTCAGCATATATGAGTTAGCTC-3' _

Sequence ID NO: 10 (Ebola VP30 forward primer):

5'-CCCATCGATCAGATCTGCGAACCGGTAGAG-3'

Sequence ID NO: 11 (Ebola VP30 reverse primer):

5'-CCCATCGATGTACCCTCATCAGACCATGAGC-3'

Sequence ID NO: 12 (Ebola VP35 forward primer):

5'-GGGATCGATAGAAAAGCTGGTCTAACAAGATGA-3'

Sequence ID NO: 13 (Ebola VP35 reverse primer):

5'-CCCATCGATCTCAÇAAGTGTATCATTAATGTAACGT-3'

Sequence ID NO: 14 (Ebola VP40 forward primer):

5'-CCCATCGATCCTACCTCGGCTGAGAGAGTG-3'

Sequence ID NO: 15 (Ebola VP40 reverse primer):

5'-CCCATCGATATGTTATGCACTATCCCTGAGAAG-3'

Sequence ID NO: 16 (Ebola VP30#2 reverse primer):

5' CCC ATC GAT CTG TTA GGG TTG TAT CATA CC -3'

Sequence ID NO: 1 (Ebola GP amino acid sequence from replicon):

Met Gly Val Thr Gly Ile Leu Gln Leu Pro Arg Asp Arg Phe Lys Arg Thr Ser
Phe Phe Leu Trp Val Ile Ile Leu Phe Gln Arg Thr Phe Ser Ile Pro Leu Gly
Val Ile His Asn Ser Thr Leu Gln Val Ser Asp Val Asp Lys Leu Val Cys Arg
Asp Lys Leu Ser Ser Thr Asn Gln Leu Arg Ser Val Gly Leu Asn Leu Glu Gly
Asn Gly Val Ala Thr Asp Val Pro Ser Ala Thr Lys Arg Trp Gly Phe Arg Ser
Gly Val Pro Pro Lys Val Val Asn Tyr Glu Ala Gly Glu Trp Ala Glu Asn Cys
Tyr Asn Leu Glu Ile Lys Lys Pro Asp Gly Ser Glu Cys Leu Pro Ala Ala Pro
Asp Gly Ile Arg Gly Phe Pro Arg Cys Arg Tyr Val His Lys Val Ser Gly Thr
Gly Pro Cys Ala Gly Asp Phe Ala Phe His Lys Glu Gly Ala Phe Phe Leu Tyr
Asp Arg Leu Ala Ser Thr Val Ile Tyr Arg Gly Thr Thr Phe Ala Glu Gly Val
Val Ala Phe Leu Ile Leu Pro Gln Ala Lys Lys Asp Phe Phe Ser Ser His Pro
Leu Arg Glu Pro Val Asn Ala Thr Glu Asp Pro Ser Ser Gly Tyr Tyr Ser Thr
Thr Ile Arg Tyr Gln Ala Thr Gly Phe Gly Thr Asn Glu Thr Glu Tyr Leu Phe
Glu Val Asp Asn Leu Thr Tyr Val Gln Leu Glu Ser Arg Phe Thr Pro Gln Phe
Leu Leu Gln Leu Asn Glu Thr Ile Tyr Thr Ser Gly Lys Arg Ser Asn Thr Thr
Gly Lys Leu Ile Trp Lys Val Asn Pro Glu Ile Asp Thr Thr Ile Gly Glu Trp
Ala Phe Trp Glu Thr Lys Lys Asn Leu Thr Arg Lys Ile Arg Ser Glu Glu Leu
Ser Phe Thr Val Val Ser Asn Gly Ala Lys Asn Ile Ser Gly Gln Ser Pro Ala
Arg Thr Ser Ser Asp Pro Gly Thr Asn Thr Thr Thr Glu Asp His Lys Ile Met
Ala Ser Glu Asn Ser Ser Ala Met Val Gln Val His Ser Gln Gly Arg Glu Ala
Ala Val Ser His Leu Thr Thr Leu Ala Thr Ile Ser Thr Ser Pro Gln Ser Leu
Thr Thr Lys Pro Gly Pro Asp Asn Ser Thr His Asn Thr Pro Val Tyr Lys Leu
Asp Ile Ser Glu Ala Thr Gln Val Glu Gln His His Arg Arg Thr Asp Asn Asp
Ser Thr Ala Ser Asp Thr Pro Ser Ala Thr Thr Ala Ala Gly Pro Pro Lys Ala
Glu Asn Thr Asn Thr Ser Lys Ser Thr Asp Phe Leu Asp Pro Ala Thr Thr Thr
Ser Pro Gln Asn His Ser Glu Thr Ala Gly Asn Asn Asn Thr His His Gln Asp
Thr Gly Glu Glu Ser Ala Ser Ser Gly Lys Leu Gly Leu Ile Thr Asn Thr Ile
Ala Gly Val Ala Gly Leu Ile Thr Gly Gly Arg Arg Thr Arg Arg Glu Ala Ile
Val Asn Ala Gln Pro Lys Cys Asn Pro Asn Leu His Tyr Trp Thr Thr Gln Asp
Glu Gly Ala Ala Ile Gly Leu Ala Trp Ile Pro Tyr Phe Gly Pro Ala Ala Glu
Gly Ile Tyr Ile Glu Gly Leu Met His Asn Gln Asp Gly Leu Ile Cys Gly Leu
Arg Gln Leu Ala Asn Glu Thr Thr Gln Ala Leu Gln Leu Phe Leu Arg Ala Thr
Thr Glu Leu Arg Thr Phe Ser Ile Leu Asn Arg Lys Ala Ile Asp Phe Leu Leu
Gln Arg Trp Gly Gly Thr Cys His Ile Leu Gly Pro Asp Cys Cys Ile Glu Pro
His Asp Trp Thr Lys Asn Ile Thr Asp Lys Ile Asp Gln Ile Ile His Asp Phe
Val Asp Lys Thr Leu Pro Asp Gln Gly Asp Asn Asp Asn Trp Trp Thr Gly Trp
Arg Gln Trp Ile Pro Ala Gly Ile Gly Val Thr Gly Val Val Ile Ala Val Ile
Ala Leu Phe Cys Ile Cys Lys Phe Val Phe *

Sequence ID NO: 18(Ebola NP amino acid sequence from replicon):

Met Asp Ser Arg Pro Gln Lys Ile Trp Met Ala Pro Ser Leu Thr Glu Ser Asp
 Met Asp Tyr His Lys Ile Leu Thr Ala Gly Leu Ser Val Gln Gln Gly Ile Val
 Arg Gln Arg Val Ile Pro Val Tyr Gln Val Asn Asn Leu Glu Glu Ile Cys Gln
 Leu Ile Ile Gln Ala Phe Glu Ala Gly Val Asp Phe Gln Glu Ser Ala Asp Ser
 Phe Leu Leu Met Leu Cys Leu His His Ala Tyr Gln Gly Asp Tyr Lys Leu Phe
 Leu Glu Ser Gly Ala Val Lys Tyr Leu Glu Gly His Gly Phe Arg Phe Glu Val
 Lys Lys Arg Asp Gly Val Lys Arg Leu Glu Glu Leu Leu Pro Ala Val Ser Ser
 Gly Lys Asn Ile Lys Arg Thr Leu Ala Ala Met Pro Glu Glu Glu Thr Thr Glu
 Ala Asn Ala Gly Gln Phe Leu Ser Phe Ala Ser Leu Phe Leu Pro Lys Leu Val
 Val Gly Glu Lys Ala Cys Leu Glu Lys Val Gln Arg Gln Ile Gln Val His Ala
 Glu Gln Gly Leu Ile Gln Tyr Pro Thr Ala Trp Gln Ser Val Gly His Met Met
 Val Ile Phe Arg Leu Met Arg Thr Asn Phe Leu Ile Lys Phe Leu Leu Ile His
 Gln Gly Met His Met Val Ala Gly His Asp Ala Asn Asp Ala Val Ile Ser Asn
 Ser Val Ala Gln Ala Arg Phe Ser Gly Leu Leu Ile Val Lys Thr Val Leu Asp
 His Ile Leu Gln Lys Thr Glu Arg Gly Val Arg Leu His Pro Leu Ala Arg Thr
 Ala Lys Val Lys Asn Glu Val Asn Ser Phe Lys Ala Ala Leu Ser Ser Leu Ala
 Lys His Gly Glu Tyr Ala Pro Phe Ala Arg Leu Leu Asn Leu Ser Gly Val Asn
 Asn Leu Glu His Gly Leu Phe Pro Gln Leu Ser Ala Ile Ala Leu Gly Val Ala
 Thr Ala His Gly Ser Thr Leu Ala Gly Val Asn Val Gly Glu Gln Tyr Gln Gln
 Leu Arg Glu Ala Ala Thr Glu Ala Glu Lys Gln Leu Gln Gln Tyr Ala Glu Ser
 Arg Glu Leu Asp His Leu Gly Leu Asp Asp Gln Glu Lys Lys Ile Leu Met Asn
 Phe His Gln Lys Lys Asn Glu Ile Ser Phe Gln Gln Thr Asn Ala Met Val Thr
 Leu Arg Lys Glu Arg Leu Ala Lys Leu Thr Glu Ala Ile Thr Ala Ala Ser Leu
 Pro Lys Thr Ser Gly His Tyr Asp Asp Asp Asp Ile Pro Phe Pro Gly Pro
 Ile Asn Asp Asp Asp Asn Pro Gly His Gln Asp Asp Asp Pro Thr Asp Ser Gln
 Asp Thr Thr Ile Pro Asp Val Val Val Asp Pro Asp Asp Gly Ser Tyr Gly Glu
 Tyr Gln Ser Tyr Ser Glu Asn Gly Met Asn Ala Pro Asp Asp Leu Val Leu Phe
 Asp Leu Asp Glu Asp Asp Glu Asp Thr Lys Pro Val Pro Asn Arg Ser Thr Lys
 Gly Gly Gln Gln Lys Asn Ser Gln Lys Gly Gln His Ile Glu Gly Arg Gln Thr
 Gln Ser Arg Pro Ile Gln Asn Val Pro Gly Pro His Arg Thr Ile His His Ala
 Ser Ala Pro Leu Thr Asp Asn Asp Arg Arg Asn Glu Pro Ser Gly Ser Thr Ser
 Pro Arg Met Leu Thr Pro Ile Asn Glu Glu Ala Asp Pro Leu Asp Asp Ala Asp
 Asp Glu Thr Ser Ser Leu Pro Pro Leu Glu Ser Asp Asp Glu Glu Gln Asp Arg
 Asp Gly Thr Ser Asn Arg Thr Pro Thr Val Ala Pro Pro Ala Pro Val Tyr Arg
 Asp His Ser Glu Lys Lys Glu Leu Pro Gln Asp Glu Gln Gln Asp Gln Asp His
 Thr Gln Glu Ala Arg Asn Gln Asp Ser Asp Asn Thr Gln Ser Glu His Ser Phe
 Glu Glu Met Tyr Arg His Ile Leu Arg Ser Gln Gly Pro Phe Asp Ala Val Leu
 Tyr Tyr His Met Met Lys Asp Glu Pro Val Val Phe Ser Thr Ser Asp Gly Lys
 Glu Tyr Thr Tyr Pro Asp Ser Leu Glu Glu Glu Tyr Pro Pro Trp Leu Thr Glu
 Lys Glu Ala Met Asn Glu Glu Asn Arg Phe Val Thr Leu Asp Gly Gln Gln Phe
 Tyr Trp Pro Val Met Asn His Lys Asn Lys Phe Met Ala Ile Leu Gln His His
 Gln *

Sequence ID NO: 19 (Ebola VP24 amino acid sequence from replicon):

Met Ala Lys Ala Thr Gly Arg Tyr Asn Leu Ile Ser Pro Lys Lys Asp Leu Glu
 Lys Gly Val Val Leu Ser Asp Leu Cys Asn Phe Leu Val Ser Gln Thr Ile Gln
 Gly Trp Lys Val Tyr Trp Ala Gly Ile Glu Phe Asp Val Thr His Lys Gly Met
 Ala Leu Leu His Arg Leu Lys Thr Asn Asp Phe Ala Pro Ala Trp Ser Met Thr
 Arg Asn Leu Phe Pro His Leu Phe Gln Asn Pro Asn Ser Thr Ile Glu Ser Pro
 Leu Trp Ala Leu Arg Val Ile Leu Ala Ala Gly Ile Gln Asp Gln Leu Ile Asp
 Gln Ser Leu Ile Glu Pro Leu Ala Gly Ala Leu Gly Leu Ile Ser Asp Trp Leu
 Leu Thr Thr Asn Thr Asn His Phe Asn Met Arg Thr Gln Arg Val Lys Glu Gln
 Leu Ser Leu Lys Met Leu Ser Leu Ile Arg Ser Asn Ile Leu Lys Phe Ile Asn
 Lys Leu Asp Ala Leu His Val Val Asn Tyr Asn Gly Leu Leu Ser Ser Ile Glu
 Ile Gly Thr Gln Asn His Thr Ile Ile Ile Thr Arg Thr Asn Met Gly Phe Leu
 Val Glu Leu Gln Glu Pro Asp Lys Ser Ala Met Asn Arg Met Lys Pro Gly Pro
 Ala Lys Phe Ser Leu Leu His Glu Ser Thr Leu Lys Ala Phe Thr Gln Gly Ser
 Ser Thr Arg Met Gln Ser Leu Ile Leu Glu Phe Asn Ser Ser Leu Ala Ile *

Sequence ID NO: 20 (Ebola VP30 amino acid sequence from replicon):

Met Glu Ala Ser Tyr Glu Arg Gly Arg Pro Arg Ala Ala Arg Gln His Ser Arg
 Asp Gly His Asp His His Val Arg Ala Arg Ser Ser Ser Arg Glu Asn Tyr Arg
 Gly Glu Tyr Arg Gln Ser Arg Ser Ala Ser Gln Val Arg Val Pro Thr Val Phe
 His Lys Lys Arg Val Glu Pro Leu Thr Val Pro Pro Ala Pro Lys Asp Ile Cys
 Pro Thr Leu Lys Lys Gly Phe Leu Cys Asp Ser Ser Phe Cys Lys Lys Asp His
 Gln Leu Glu Ser Leu Thr Asp Arg Glu Leu Leu Leu Leu Ile Ala Arg Lys Thr
 Cys Gly Ser Val Glu Gln Gln Leu Asn Ile Thr Ala Pro Lys Asp Ser Arg Leu
 Ala Asn Pro Thr Ala Asp Asp Phe Gln Gln Glu Glu Gly Pro Lys Ile Thr Leu
 Leu Thr Leu Ile Lys Thr Ala Glu His Trp Ala Arg Gln Asp Ile Arg Thr Ile
 Glu Asp Ser Lys Leu Arg Ala Leu Leu Thr Leu Cys Ala Val Met Thr Arg Lys
 Phe Ser Lys Ser Gln Leu Ser Leu Leu Cys Glu Thr His Leu Arg Arg Glu Gly
 Leu Gly Gln Asp Gln Ala Glu Pro Val Leu Glu Val Tyr Gln Arg Leu His Ser
 Asp Lys Gly Gly Ser Phe Glu Ala Ala Leu Trp Gln Gln Trp Asp Leu Gln Ser
 Leu Ile Met Phe Ile Thr Ala Phe Leu Asn Ile Ala Leu Gln Leu Pro Cys Glu
 Ser Ser Ala Val Val Val Ser Gly Leu Arg Thr Leu Val Pro Gln Ser Asp Asn
 Glu Glu Ala Ser Thr Asn Pro Gly Thr Cys Ser Trp Ser Asp Glu Gly Thr Ser
 Ile Gln Gln Gln Leu Ala Ser Cys Leu His Arg Thr Arg Gly Asp Trp His Ala
 Ala Leu Lys Phe Leu Phe Tyr Phe Ser Phe Leu Phe Arg Ile Gly Phe Cys Phe
 *

Sequence ID NO: 21 (Ebola VP35 amino acid sequence from replicon):

Met Thr Thr Arg Thr Lys Gly Arg Gly His Thr Ala Ala Thr Thr Gln Asn Asp
 Arg Met Pro Gly Pro Glu Leu Ser Gly Trp Ile Ser Glu Gln Leu Met Thr Gly
 Arg Ile Pro Val Ser Asp Ile Phe Cys Asp Ile Glu Asn Asn Pro Gly Leu Cys
 Tyr Ala Ser Gln Met Gln Gln Thr Lys Pro Asn Pro Lys Thr Arg Asn Ser Gln
 Thr Gln Thr Asp Pro Ile Cys Asn His Ser Phe Glu Glu Val Val Gln Thr Leu
 Ala Ser Leu Ala Thr Val Val Gln Gln Gln Thr Ile Ala Ser Glu Ser Leu Glu
 Gln Arg Ile Thr Ser Leu Glu Asn Gly Leu Lys Pro Val Tyr Asp Met Ala Lys
 Thr Ile Ser Ser Leu Asn Arg Val Cys Ala Glu Met Val Ala Lys Tyr Asp Leu
 Leu Val Met Thr Thr Gly Arg Ala Thr Ala Thr Ala Ala Thr Glu Ala Tyr
 Trp Ala Glu His Gly Gln Pro Pro Gly Pro Ser Leu Tyr Glu Glu Ser Ala
 Ile Arg Gly Lys Ile Glu Ser Arg Asp Glu Thr Val Pro Gln Ser Val Arg Glu
 Ala Phe Asn Asn Leu Asn Ser Thr Thr Ser Leu Thr Glu Glu Asn Phe Gly Lys
 Pro Asp Ile Ser Ala Lys Asp Leu Arg Asn Ile Met Tyr Asp His Leu Pro Gly
 Phe Gly Thr Ala Phe His Gln Leu Val Gln Val Ile Cys Lys Leu Gly Lys Asp
 Ser Asn Ser Leu Asp Ile Ile His Ala Glu Phe Gln Ala Ser Leu Ala Glu Gly
 Asp Ser Pro Gln Cys Ala Leu Ile Gln Ile Thr Lys Arg Val Pro Ile Phe Gln
 Asp Ala Ala Pro Pro Val Ile His Ile Arg Ser Arg Gly Asp Ile Pro Arg Ala
 Cys Gln Lys Ser Leu Arg Pro Val Pro Pro Ser Pro Lys Ile Asp Arg Gly Trp
 Val Cys Val Phe Gln Leu Gln Asp Gly Lys Thr Leu Gly Leu Lys Ile *

Sequence ID NO: 22 (Ebola VP40 amino acid sequence from replicon):

Met Arg Arg Val Ile Leu Pro Thr Ala Pro Pro Glu Tyr Met Glu Ala Ile Tyr
 Pro Val Arg Ser Asn Ser Thr Ile Ala Arg Gly Gly Asn Ser Asn Thr Gly Phe
 Leu Thr Pro Glu Ser Val Asn Gly Asp Thr Pro Ser Asn Pro Leu Arg Pro Ile
 Ala Asp Asp Thr Ile Asp His Ala Ser His Thr Pro Gly Ser Val Ser Ser Ala
 Phe Ile Leu Glu Ala Met Val Asn Val Ile Ser Gly Pro Lys Val Leu Met Lys
 Gln Ile Pro Ile Trp Leu Pro Leu Gly Val Ala Asp Gln Lys Thr Tyr Ser Phe
 Asp Ser Thr Thr Ala Ala Ile Met Leu Ala Ser Tyr Thr Ile Thr His Phe Gly
 Lys Ala Thr Asn Pro Leu Val Arg Val Asn Arg Leu Gly Pro Gly Ile Pro Asp
 His Pro Leu Arg Leu Leu Arg Ile Gly Asn Gln Ala Phe Leu Gln Glu Phe Val
 Leu Pro Pro Val Gln Leu Pro Gln Tyr Phe Thr Phe Asp Leu Thr Ala Leu Lys
 Leu Ile Thr Gln Pro Leu Pro Ala Ala Thr Trp Thr Asp Asp Thr Pro Thr Gly
 Ser Asn Gly Ala Leu Arg Pro Gly Ile Ser Phe His Pro Lys Leu Arg Pro Ile
 Leu Leu Pro Asn Lys Ser Gly Lys Lys Gly Asn Ser Ala Asp Leu Thr Ser Pro
 Glu Lys Ile Gln Ala Ile Met Thr Ser Leu Gln Asp Phe Lys Ile Val Pro Ile
 Asp Pro Thr Lys Asn Ile Met Gly Ile Glu Val Pro Glu Thr Leu Val His Lys
 Leu Thr Gly Lys Lys Val Thr Ser Lys Asn Gly Gln Pro Ile Ile Pro Val Leu
 Leu Pro Lys Tyr Ile Gly Leu Asp Pro Val Ala Pro Gly Asp Leu Thr Met Val
 Ile Thr Gln Asp Cys Asp Thr Cys His Ser Pro Ala Ser Leu Pro Ala Val Ile
 Glu Lys *

Sequence ID NO: ^{#2}23 (new Ebola VP30 amino acid sequence from replicon):

Met Glu Ala Ser Tyr Glu Arg Gly Arg Pro Arg Ala Ala Arg Gln His Ser Arg
 Asp Gly His Asp His His Val Arg Ala Arg Ser Ser Ser Arg Glu Asn Tyr Arg
 Gly Glu Tyr Arg Gln Ser Arg Ser Ala Ser Gln Val Arg Val Pro Thr Val Phe
 His Lys Lys Arg Val Glu Pro Leu Thr Val Pro Pro Ala Pro Lys Asp Ile Cys
 Pro Thr Leu Lys Lys Gly Phe Leu Cys Asp Ser Ser Phe Cys Lys Lys Asp His
 Gln Leu Glu Ser Leu Thr Asp Arg Glu Leu Leu Leu Leu Ile Ala Arg Lys Thr
 Cys Gly Ser Val Glu Gln Gln Leu Asn Ile Thr Ala Pro Lys Asp Ser Arg Leu
 Ala Asn Pro Thr Ala Asp Asp Phe Gln Gln Glu Glu Gly Pro Lys Ile Thr Leu
 Leu Thr Leu Ile Lys Thr Ala Glu His Trp Ala Arg Gln Asp Ile Arg Thr Ile
 Glu Asp Ser Lys Leu Arg Ala Leu Leu Thr Leu Cys Ala Val Met Thr Arg Lys
 Phe Ser Lys Ser Gln Leu Ser Leu Leu Cys Glu Thr His Leu Arg Arg Glu Gly
 Leu Gly Gln Asp Gln Ala Glu Pro Val Leu Glu Val Tyr Gln Arg Leu His Ser
 Asp Lys Gly Gly Ser Phe Glu Ala Ala Leu Trp Gln Gln Trp Asp Arg Gln Ser
 Leu Ile Met Phe Ile Thr Ala Phe Leu Asn Ile Ala Leu Gln Leu Pro Cys Glu
 Ser Ser Ala Val Val Val Ser Gly Leu Arg Thr Leu Val Pro Gln Ser Asp Asn
 Glu Glu Ala Ser Thr Asn Pro Gly Thr Cys Ser Trp Ser Asp Glu Gly Thr Pro
 *

Sequence ID NO: ²⁴24 (Ebola NP CTL epitope):

VYQVNNLEEIC

Sequence ID NO: ²⁵25 (Ebola VP24 CTL epitope):

LKFINKLDALLVVNYNGLLSSIF



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(57) Abstract

Using the Ebola GP, NP, VP24, VP30, VP35 and VP40 virion proteins, a method and composition for use in inducing an immune response which is protective against infection with Ebola virus is described.

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1 **TITLE OF THE INVENTION**

2

3 Ebola Virion Proteins Expressed from Venezuelan Equine

4 Encephalitis (VEE) Virus Replicons

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11 **INTRODUCTION**

12

13 Ebola viruses, members of the family

14 Filoviridae, are associated with outbreaks of highly

15 lethal hemorrhagic fever in humans and nonhuman

16 primates. The natural reservoir of the virus is

17 unknown and there currently are no available vaccines

18 or effective therapeutic treatments for filovirus

19 infections. The genome of Ebola virus consists of a

20 single strand of negative sense RNA that is

21 approximately 19 kb in length. This RNA contains seven

22 sequentially arranged genes that produce 8 mRNAs upon

23 infection (Fig. 1). Ebola virions, like virions of

24 other filoviruses, contain seven proteins: a surface

25 glycoprotein (GP), a nucleoprotein (NP), four virion

26 structural proteins (VP40, VP35, VP30, and VP24), and

27 an RNA-dependent RNA polymerase (L) (Feldmann et

28 al. (1992) *Virus Res.* **24**, 1-19; Sanchez et al., (1993)

29 *Virus Res.* **29**, 215-240; reviewed in Peters et al.

30 (1996) In Fields Virology, Third ed. pp. 1161-1176.

31 Fields, B. N., Knipe, D. M., Howley, P.M., et al. eds.

32 Lippincott-Raven Publishers, Philadelphia). The

33 glycoprotein of Ebola virus is unusual in that it is

34 encoded in two open reading frames. Transcriptional

35 editing is needed to express the transmembrane form

36 that is incorporated into the virion (Sanchez et al.

37 (1996) *Proc. Natl. Acad. Sci. USA* **93**, 3602-3607;

1 Volchkov et al, (1995) *Virology* **214**, 421-430. The
2 unedited form produces a nonstructural secreted
3 glycoprotein (sGP) that is synthesized in large
4 amounts early during the course of infection. Little
5 is known about the biological functions of these
6 proteins and it is not known which antigens
7 significantly contribute to protection and should
8 therefore be used to induce an immune response.

9 Recent studies using rodent models to evaluate
10 subunit vaccines for Ebola virus infection using
11 recombinant vaccinia virus encoding Ebola virus GP
12 (Gilligan et al., (1997) *In Vaccines* **97**, pp. 87-92.
13 Cold Spring Harbor Laboratory Press, Cold Spring
14 Harbor, N.Y.), or naked DNA constructs expressing
15 either GP or sGP (Xu et al. (1998) *Nature Med.* **4**, 37-
16 42) have demonstrated the protective efficacy of Ebola
17 virus GP in guinea pigs. (All documents cited herein
18 *supra* and *infra* are hereby incorporated in their
19 entirety by reference thereto.) Additionally, Ebola
20 virus NP and GP genes expressed from naked DNA
21 vaccines (Vanderzanden et al., (1998) *Virology* **246**,
22 134-144) have elicited protective immunity in BALB/c
23 mice. However, successful vaccination of nonhuman
24 primates with individual Ebola virus genes has not
25 been demonstrated. Therefore, there exists a need for
26 a vaccine which is efficacious for protection from
27 Ebola virus infection.

28

29 SUMMARY OF THE INVENTION

30 The present invention satisfies the need
31 discussed above. The present invention relates to a
32 method and composition for use in inducing an immune
33 response which is protective against infection with
34 Ebola virus.

35 Because the biological functions of the
36 individual Ebola virus proteins are not known and the
37 immune mechanisms necessary for preventing and

1 clearing Ebola virus infection are not well
2 understood, it was not clear which antigens
3 significantly contribute to protection and should
4 therefore be included in an eventual vaccine candidate
5 to induce a protective immune response. We evaluated
6 the ability of packaged Venezuelan equine encephalitis
7 (VEE) virus replicons expressing GP, NP, VP40, VP35,
8 VP30 and VP24 virion proteins of Ebola virus to elicit
9 protective immunity in two strains of mice which
10 differ at the major histocompatibility locus. There
11 are no published reports of the VP proteins having
12 been assayed as antigens for the production of an
13 immune response in a mammal.

14 The VEE virus replicon (Vrep) is a genetically
15 reorganized version of the VEE virus genome in which
16 the structural protein genes are replaced with a gene
17 from an immunogen of interest, such as the Ebola virus
18 virion proteins. This replicon can be transcribed to
19 produce a self-replicating RNA that can be packaged
20 into infectious particles using defective helper RNAs
21 that encode the glycoprotein and capsid proteins of
22 the VEE virus. Since the packaged replicons do not
23 encode the structural proteins, they are incapable of
24 spreading to new cells and therefore undergo a single
25 abortive round of replication in which large amounts
26 of the inserted immunogen are made in the infected
27 cells. The VEE virus replicon system is described in
28 U.S. Patent to Johnston *et al.*, patent no. 5,792,462
29 issued on August 11, 1998.

30 For our purposes, each of the Ebola virus genes
31 were individually inserted into a VEE virus replicon
32 vector. The VP24, VP30, VP35, and VP40 genes of Ebola
33 Zaire 1976 (Mayinga isolate) were cloned by reverse
34 transcription of RNA from Ebola-infected Vero E6 cells
35 and viral cDNAs were amplified using the polymerase
36 chain reaction. The Ebola Zaire 1976 (Mayinga isolate)
37 GP and NP genes were obtained from plasmids already
38 containing these genes (Sanchez, A. *et al.*, (1989)

1 *Virology* **170**, 81-91; Sanchez, A. et al., (1993) *Virus*
2 *Res.* **29**, 215-240) and were subcloned into the VEE
3 replicon vector.

4 After characterization of the Ebola gene
5 products expressed from the VEE replicon constructs in
6 cell culture, these constructs were packaged into
7 infectious VEE virus replicon particles (VRPs) and
8 subcutaneously injected into BALB/c and C57BL/6 mice.
9 As controls in these experiments, mice were also
10 immunized with a VEE replicon expressing Lassa
11 nucleoprotein (NP) as an irrelevant control antigen,
12 or injected with PBS buffer alone. The results of this
13 study demonstrate that VRPs expressing the Ebola GP,
14 NP, VP24, VP30, VP35 or VP40 genes induced protection
15 in mice and may provide protection in humans.

16

17 Therefore, it is one object of the present
18 invention to provide a DNA fragment encoding each of
19 the Ebola Zaire 1976 GP, NP, VP24, VP30, VP35, and
20 VP40 virion proteins (SEQUENCE ID NOS. 1-7).

21

22 It is another object of the present invention to
23 provide the DNA fragments of Ebola virion proteins in
24 a recombinant vector. When the vector is an
25 expression vector, the Ebola virion proteins GP, NP,
26 VP24, VP30, VP35, and VP40 are produced.

27

28 It is yet another object of the present
29 invention to provide a VEE virus replicon vector
30 comprising a VEE virus replicon and a DNA fragment
31 encoding any of the Ebola Zaire 1976 (Mayinga isolate)
32 GP, NP, VP24, VP30, VP35, or VP40 proteins. The
33 construct can be used as a nucleic acid vaccine or for
34 the production of self replicating RNA.

35

36 It is another object of the present invention to
37 provide a self replicating RNA comprising the VEE
38 virus replicon and any of the Ebola Zaire 1976

1 (Mayinga isolate) RNAs encoding the GP, NP, VP24,
2 VP30, VP35, and VP40 proteins described above. The
3 RNA can be used as a vaccine for protection from Ebola
4 infection. When the RNA is packaged, a VEE virus
5 replicon particle is produced.

6

7 It is another object of the present invention to
8 provide infectious VEE virus replicon particles
9 produced from the VEE virus replicon RNAs described
10 above.

11

12 It is further an object of the invention to
13 provide an immunological composition for the
14 protection of subjects against Ebola virus infection,
15 comprising VEE virus replicon particles containing the
16 Ebola virus GP, NP, VP24, VP30, VP35, or VP40
17 proteins, or any combination of different VEE virus
18 replicons each containing one or more different Ebola
19 proteins selected from GP, NP, VP24, VP30, VP35 and
20 VP40.

21

22 BRIEF DESCRIPTION OF THE DRAWINGS

23 These and other features, aspects, and
24 advantages of the present invention will become better
25 understood with reference to the following description
26 and appended claims, and accompanying drawings where:

27 Figure 1 is a schematic description of the
28 organization of the Ebola virus genome.

29 Figures 2A, 2B and 2C are schematic
30 representations of the VEE replicon constructs
31 containing Ebola genes.

32 Figure 3 shows the generation of VEE viral-like
33 particles containing Ebola genes.

34 Figure 4 is an immunoprecipitation of Ebola
35 proteins produced from replicon constructs.

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DETAILED DESCRIPTION

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In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

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Filoviruses. The filoviruses (e.g. Ebola Zaire 1976) cause acute hemorrhagic fever characterized by high mortality. Humans can contract filoviruses by infection in endemic regions, by contact with imported primates, and by performing scientific research with the virus. However, there currently are no available vaccines or effective therapeutic treatments for filovirus infection. The virions of filoviruses contain seven proteins: a membrane-anchored glycoprotein (GP), a nucleoprotein (NP), an RNA-dependent RNA polymerase (L), and four virion structural proteins (VP24, VP30, VP35, and VP40). Little is known about the biological functions of these proteins and it is not known which antigens significantly contribute to protection and should therefore be used in an eventual vaccine candidate.

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Replicon. A replicon is equivalent to a full-length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be inserted downstream of the 26S promoter into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be inserted into this cloning site. The RNA that is transcribed from the replicon is capable of replicating and expressing viral proteins in a manner that is similar to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed from

1 the 26S promoter in the replicon. This system does not
2 yield any progeny virus particles because there are no
3 viral structural proteins available to package the RNA
4 into particles.

5 Particles which appear structurally identical to
6 virus particles can be produced by supplying
7 structural protein RNAs *in trans* for packaging of the
8 replicon RNA. This is typically done with two
9 defective helper RNAs which encode the structural
10 proteins. One helper consists of a full length
11 infectious clone from which the nonstructural protein
12 genes and the glycoprotein genes are deleted. This
13 helper retains only the terminal nucleotide sequences,
14 the promoter for subgenomic mRNA transcription and the
15 sequences for the viral nucleocapsid protein. The
16 second helper is identical to the first except that
17 the nucleocapsid gene is deleted and only the
18 glycoprotein genes are retained. The helper RNAs are
19 transcribed *in vitro* and are co-transfected with
20 replicon RNA. Because the replicon RNA retains the
21 sequences for packaging by the nucleocapsid protein,
22 and because the helpers lack these sequences, only the
23 replicon RNA is packaged by the viral structural
24 proteins. The packaged replicon particles are released
25 from the host cell and can then be purified and
26 inoculated into animals. The packaged replicon
27 particles will have a tropism similar to the parent
28 virus. The packaged replicon particles will infect
29 cells and initiate a single round of replication,
30 resulting in the expression of only the virus
31 nonstructural proteins and the product of the
32 heterologous gene that was cloned in the place of the
33 virus structural proteins. In the absence of RNA
34 encoding the virus structural proteins, no progeny
35 virus particles can be produced from the cells
36 infected by packaged replicon particles.

37 The Venezuelan equine encephalitis (VEE) virus
38 replicon is a genetically reorganized version of the

1 VEE virus genome in which the genes encoding the VEE
2 structural proteins are replaced with a heterologous
3 gene of interest. In the present invention, the
4 heterologous genes are the GP, NP, or VP virion
5 proteins from the Ebola virus. The result is a self-
6 replicating RNA that can be packaged into infectious
7 particles using defective helper RNAs that encode the
8 glycoprotein and capsid proteins of the VEE virus. The
9 replicon and its use is further described in U.S.
10 Patent no 5,792,462 issued to Johnston et al. on
11 August 11, 1998.

12 **Subject.** Includes both human, animal, e.g.,
13 horse, donkey, pig, mouse, hamster, monkey, chicken,
14 and insect such as mosquito.

15 In one embodiment, the present invention relates
16 to DNA fragments which encode any of the Ebola Zaire
17 1976 (Mayinga isolate) GP, NP, VP24, VP30, VP35, and
18 VP40 proteins. The GP and NP genes of Ebola Zaire were
19 previously sequenced by Sanchez et al. (1993, *supra*)
20 and have been deposited in GenBank (accession number
21 L11365). A plasmid encoding the VEE replicon vector
22 containing a unique ClaI site downstream from the 26S
23 promoter was described previously (Davis, N. L. et
24 al., (1996) *J. Virol.* **70**, 3781-3787; Pushko, P. et
25 al. (1997) *Virology* **239**, 389-401). The Ebola GP and
26 NP genes from the Ebola Zaire 1976 virus were derived
27 from PS64- and PGEM3ZF(-)-based plasmids (Sanchez, A.
28 et al. (1989) *Virology* **170**, 81-91; Sanchez, A. et al.
29 (1993) *Virus Res.* **29**, 215-240). From these plasmids,
30 the BamHI-EcoRI (2.3 kb) and BamHI-KpnI (2.4 kb)
31 fragments containing the NP and GP genes,
32 respectively, were subcloned into a shuttle vector
33 that had been digested with BamHI and EcoRI (Davis et
34 al. (1996) *supra*; Grieder, F. B. et al. (1995)
35 *Virology* **206**, 994-1006). For cloning of the GP gene,
36 overhanging ends produced by KpnI (in the GP fragment)
37 and EcoRI (in the shuttle vector) were made blunt by
38 incubation with T4 DNA polymerase according to methods

1 known in the art. From the shuttle vector, GP or NP
2 genes were subcloned as ClaI-fragments into the ClaI
3 site of the replicon clone, resulting in plasmids
4 encoding the GP or NP genes in place of the VEE
5 structural protein genes downstream from the VEE 26S
6 promoter.

7 The VP genes of Ebola Zaire were previously
8 sequenced by Sanchez *et al.* (1993, *supra*) and have
9 been deposited in GenBank (accession number L11365).
10 The VP genes of Ebola used in the present invention
11 were cloned by reverse transcription of RNA from
12 Ebola-infected Vero E6 cells and subsequent
13 amplification of viral cDNAs using the polymerase
14 chain reaction. First strand synthesis was primed with
15 oligo dT (Life Technologies). Second strand synthesis
16 and subsequent amplification of viral cDNAs were
17 performed with gene-specific primers (SEQ ID NOS:8-
18 16). The primer sequences were derived from the
19 GenBank deposited sequences and were designed to
20 contain a ClaI restriction site for cloning the
21 amplified VP genes into the ClaI site of the replicon
22 vector. The letters and numbers in bold print indicate
23 Ebola gene sequences in the primers and the
24 corresponding location numbers based on the GenBank
25 deposited sequences.

26 VP24: (1) forward primer is

27 5'-GGGATCGATCTCCAGACACCAAGCAAGACC-3' (SEQ ID NO:8)

28 (10,311-10,331)

29 (2) reverse primer is

30 5'-GGGATCGATGAGTCAGCATATATGAGTTAGCTC-3' (SEQ ID

31 NO:9)

32 (11,122-11,145)

33 VP30: (1) forward primer is

34 5'-CCCATCGATCAGATCTGCGAACCGGTAGAG-3' SEQ ID NO:10)

35 (8408-8430)

36 (2) reverse primer is

37 5'-CCCATCGATGTACCCTCATCAGACCATGAGC-3' (SEQ ID

38 NO:11)

1 (9347-9368)

2 VP35: (1) forward primer is

3 5'-GGGATCGATAGAAAAGCTGGTCTAACAAGATGA-3' (SEQ ID

4 NO:12)

5 (3110-3133)

6 (2) reverse primer is

7 5'-CCCATCGATCTCACAAAGTGATCATTAATGTAACGT-3' (SEQ ID

8 NO:13) (4218-4244)

9 VP40: (1) forward primer is

10 5'-CCCATCGATCCTACCTCGGCTGAGAGAGTG-3' (SEQ ID NO:14)

11 (4408-4428)

12 (2) reverse primer is

13 5'-CCCATCGATATGTTATGCACTATCCCTGAGAAG-3' (SEQ ID

14 NO:15)

15 (5495-5518)

16 VP30 #2:

17 (1) forward primer as for VP30 above

18 (2) reverse primer is

19 5'-CCCATCGATCTGTTAGGGTTGTATCATACC-3' (SEQ ID NO:16)

20

21 The Ebola virus genes cloned into the VEE

22 replicon were sequenced. Changes in the DNA sequence

23 relative to the sequence published by Sanchez *et al.*

24 (1993) are described relative to the nucleotide (nt)

25 sequence number from GenBank (accession number

26 L11365).

27 The nucleotide sequence we obtained for Ebola

28 virus GP (SEQ ID NO:1) differed from the GenBank

29 sequence by a transition from A to G at nt 8023. This

30 resulted in a change in the amino acid sequence from

31 Ile to Val at position 662 (SEQ ID NO: 17).

32 The nucleotide sequence we obtained for Ebola

33 virus NP (SEQ ID NO:2) differed from the GenBank

34 sequence at the following 4 positions: insertion of a

35 C residue between nt 973 and 974, deletion of a G

36 residue at nt 979, transition from C to T at nt 1307,

37 and a transversion from A to C at nt 2745. These

38 changes resulted in a change in the protein sequence

1 from Arg to Glu at position 170 and a change from Leu
2 to Phe at position 280 (SEQ ID NO: 18).

3 The Ebola virus VP24 nucleotide sequence (SEQ ID
4 NO:3) differed from the GenBank sequence at 6
5 positions, resulting in 3 nonconservative changes in
6 the amino acid sequence. The changes in the DNA
7 sequence of VP24 consisted of a transversion from G to
8 C at nt 10795, a transversion from C to G at nt 10796,
9 a transversion from T to A at nt 10846, a transversion
10 from A to T at nt 10847, a transversion from C to G at
11 nt 11040, and a transversion from C to G at nt 11041.
12 The changes in the amino acid sequence of VP24
13 consisted of a Cys to Ser change at position 151, a
14 Leu to His change at position 168, and a Pro to Gly
15 change at position 233 (SEQ ID NO: 19).

16 Two different sequences for the Ebola virus VP30
17 gene, VP30 and VP30#2 (SEQ ID NOS: 4 and 7) are
18 included. Both of these sequences differ from the
19 GenBank sequence by the insertion of an A residue in
20 the upstream noncoding sequence between nt 8469 and
21 8470 and an insertion of a T residue between nt 9275
22 and 9276 that results in a change in the open reading
23 frame of VP30 and VP30#2 after position 255 (SEQ ID
24 NOS: 20 and 23). As a result, the C-terminus of the
25 VP30 protein differs significantly from that
26 previously reported. In addition to these 2 changes,
27 the VP30#2 nucleic acid in SEQ ID NO:7 contains a
28 conservative transition from T to C at nt 9217.
29 Because the primers originally used to clone the VP30
30 gene into the replicon were designed based on the
31 GenBank sequence, the first clone that we constructed
32 (SEQ ID NO: 4) did not contain what we believe to be
33 the authentic C-terminus of the protein. Therefore,
34 in the absence of the VP30 stop codon, the C-terminal
35 codon was replaced with 37 amino acids derived from
36 the vector sequence. The resulting VP30 construct
37 therefore differed from the GenBank sequence in that
38 it contained 32 amino acids of VP30 sequence

1 (positions 256 to 287, SEQ ID NO:20) and 37 amino
2 acids of irrelevant sequence (positions 288 to 324,
3 SEQ ID NO:20) in the place of the C-terminal 5 amino
4 acids reported in GenBank. However, inclusion of 37
5 amino acids of vector sequence in place of the C-
6 terminal amino acid (Pro, SEQ ID NO: 23) did not
7 inhibit the ability of the protein to serve as a
8 protective antigen in BALB/c mice. We are currently
9 examining the ability of the new VEE replicon
10 construct, which we believe contains the authentic C-
11 terminus of VP30 (VP30#2, SEQ ID NO: 23), to protect
12 mice against a lethal Ebola challenge.

13 The nucleotide sequence for Ebola virus VP35 (SEQ
14 ID NO:5) differed from the GenBank sequence by a
15 transition from T to C at nt 4006, a transition from T
16 to C at nt 4025, and an insertion of a T residue
17 between nt 4102 and 4103. These sequence changes
18 resulted in a change from a Ser to a Pro at position
19 293 and a change from Phe to Ser at position 299 (SEQ
20 ID NO: 21). The insertion of the T residue resulted
21 in a change in the open reading frame of VP35 from
22 that previously reported by Sanchez *et al.* (1993)
23 following amino acid number 324. As a result, Ebola
24 virus VP35 encodes a protein of 340 amino acids, where
25 amino acids 325 to 340 (SEQ ID NO: 21) differ from and
26 replace the C-terminal 27 amino acids of the
27 previously published sequence.

28 Sequencing of VP30 and VP35 was also performed
29 on RT/PCR products from RNA derived from cells that
30 were infected with Ebola virus 1976,, Ebola virus 1995
31 or the mouse-adapted Ebola virus. The changes noted
32 above for the Vrep constructs were also found in these
33 Ebola viruses. Thus, we believe that these changes are
34 real events and not artifacts of cloning.

35 The Ebola virus VP40 nucleotide sequence (SEQ ID
36 NO:6) differed from the GenBank sequence by a
37 transversion from a C to G at nt 4451 and a transition
38 from a G to A at nt 5081. These sequence changes did

1 not alter the protein sequence of VP40 (SEQ ID NO: 22)
2 from that of the published sequence.

3 DNA or polynucleotide sequences to which the
4 invention also relates include sequences of at least
5 about 6 nucleotides, preferably at least about 8
6 nucleotides, more preferably at least about 10-12
7 nucleotides, most preferably at least about 15-20
8 nucleotides corresponding, i.e., homologous to or
9 complementary to, a region of the Ebola nucleotide
10 sequences described above. Preferably, the sequence of
11 the region from which the polynucleotide is derived is
12 homologous to or complementary to a sequence which is
13 unique to the Ebola genes. Whether or not a sequence is
14 unique to the Ebola gene can be determined by techniques
15 known to those of skill in the art. For example, the
16 sequence can be compared to sequences in databanks,
17 e.g., GenBank and compared by DNA:DNA hybridization.
18 Regions from which typical DNA sequences may be derived
19 include but are not limited to, for example, regions
20 encoding specific epitopes, as well as non-transcribed
21 and/or non-translated regions.

22 The derived polynucleotide is not necessarily
23 physically derived from the nucleotide sequences shown
24 in SEQ ID NO:1-7, but may be generated in any manner,
25 including for example, chemical synthesis or DNA
26 replication or reverse transcription or transcription,
27 which are based on the information provided by the
28 sequence of bases in the region(s) from which the
29 polynucleotide is derived. In addition, combinations
30 of regions corresponding to that of the designated
31 sequence may be modified in ways known in the art to
32 be consistent with an intended use. The sequences of
33 the present invention can be used in diagnostic assays
34 such as hybridization assays and polymerase chain
35 reaction assays, for example, for the discovery of
36 other Ebola sequences.

37 In another embodiment, the present invention
38 relates to a recombinant DNA molecule that includes a

1 vector and a DNA sequence as described above. The
2 vector can take the form of a plasmid, a eukaryotic
3 expression vector such as pcDNA3.1, pRcCMV2,
4 pZeoSV2, or pCDM8, which are available from Invitrogen,
5 or a virus vector such as baculovirus vectors,
6 retrovirus vectors or adenovirus vectors, alphavirus
7 vectors, and others known in the art.

8 In a further embodiment, the present invention
9 relates to host cells stably transformed or
10 transfected with the above-described recombinant DNA
11 constructs. The host cell can be prokaryotic (for
12 example, bacterial), lower eukaryotic (for example,
13 yeast or insect) or higher eukaryotic (for example,
14 all mammals, including but not limited to mouse and
15 human). Both prokaryotic and eukaryotic host cells may
16 be used for expression of the desired coding sequences
17 when appropriate control sequences which are
18 compatible with the designated host are used.

19 Among prokaryotic hosts, *E. coli* is the most
20 frequently used host cell for expression. General
21 control sequences for prokaryotes include promoters
22 and ribosome binding sites. Transfer vectors
23 compatible with prokaryotic hosts are commonly derived
24 from a plasmid containing genes conferring ampicillin
25 and tetracycline resistance (for example, pBR322) or
26 from the various pUC vectors, which also contain
27 sequences conferring antibiotic resistance. These
28 antibiotic resistance genes may be used to obtain
29 successful transformants by selection on medium
30 containing the appropriate antibiotics. Please see
31 e.g., Maniatis, Fritsch and Sambrook, Molecular
32 Cloning; A Laboratory Manual (1982) or DNA Cloning,
33 Volumes I and II (D. N. Glover ed. 1985) for general
34 cloning methods. The DNA sequence can be present in
35 the vector operably linked to sequences encoding an
36 IgG molecule, an adjuvant, a carrier, or an agent for

1 aid in purification of Ebola proteins, such as
2 glutathione S-transferase.

3 In addition, the Ebola virus gene products can
4 also be expressed in eukaryotic host cells such as
5 yeast cells and mammalian cells. *Saccharomyces*
6 *cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia*
7 *pastoris* are the most commonly used yeast hosts.
8 Control sequences for yeast vectors are known in the
9 art. Mammalian cell lines available as hosts for
10 expression of cloned genes are known in the art and
11 include many immortalized cell lines available from
12 the American Type Culture Collection (ATCC), such as
13 CHO cells, Vero cells, baby hamster kidney (BHK) cells
14 and COS cells, to name a few. Suitable promoters are
15 also known in the art and include viral promoters such
16 as that from SV40, Rous sarcoma virus (RSV),
17 adenovirus (ADV), bovine papilloma virus (BPV), and
18 cytomegalovirus (CMV). Mammalian cells may also
19 require terminator sequences, poly A addition
20 sequences, enhancer sequences which increase
21 expression, or sequences which cause amplification of
22 the gene. These sequences are known in the art.

23 The transformed or transfected host cells can be
24 used as a source of DNA sequences described above.
25 When the recombinant molecule takes the form of an
26 expression system, the transformed or transfected
27 cells can be used as a source of the protein described
28 below.

29 In another embodiment, the present invention
30 relates to Ebola virion proteins such as GP having an
31 amino acid sequence corresponding to SEQ ID NO:17
32 encompassing 676 amino acids, NP, having an amino acid
33 sequence corresponding to SEQ ID NO:18 encompassing
34 739 amino acids, VP24, having an amino acid sequence
35 corresponding to SEQ ID NO:19 encompassing 251 amino
36 acids, VP30, having an amino acid sequence
37 corresponding to SEQ ID NO:20 encompassing 324 amino
38 acids, VP35, having an amino acid sequence

1 corresponding to SEQ ID NO:21 encompassing 340 amino
2 acids, and VP40, having an amino acid sequence
3 corresponding to SEQ ID NO:22, encompassing 326 amino
4 acids, and VP30#2, having an amino acid sequence
5 corresponding to SEQ ID NO:23 encompassing 288 amino
6 acids, or any allelic variation of the amino acid
7 sequences. By allelic variation is meant a natural or
8 synthetic change in one or more amino acids which
9 occurs between different serotypes or strains of Ebola
10 virus and does not affect the antigenic properties of
11 the protein. There are different strains of Ebola
12 (Zaire 1976, Zaire 1995, Reston, Sudan, and Ivory
13 Coast). The NP and VP genes of these different viruses
14 have not been sequenced. It would be expected that
15 these proteins would have homology among different
16 strains and that vaccination against one Ebola virus
17 strain might afford cross protection to other Ebola
18 virus strains.

19 A polypeptide or amino acid sequence derived
20 from any of the amino acid sequences in SEQ ID NO:17,
21 18, 19, 20, 21, 22, and 23 refers to a polypeptide
22 having an amino acid sequence identical to that of a
23 polypeptide encoded in the sequence, or a portion
24 thereof wherein the portion consists of at least 2-5
25 amino acids, preferably at least 8-10 amino acids, and
26 more preferably at least 11-15 amino acids, or which
27 is immunologically identifiable with a polypeptide
28 encoded in the sequence.

29 A recombinant or derived polypeptide is not
30 necessarily translated from a designated nucleic acid
31 sequence, or the DNA sequence found in GenBank
32 accession number L11365. It may be generated in any
33 manner, including for example, chemical synthesis, or
34 expression from a recombinant expression system.

35 When the DNA or RNA sequences described above
36 are in a replicon expression system, such as the VEE
37 replicon described above, the proteins can be
38 expressed *in vivo*. The DNA sequence for any of the

1 GP, NP, VP24, VP30, VP35, and VP40 virion proteins can
2 be cloned into the multiple cloning site of a replicon
3 such that transcription of the RNA from the replicon
4 yields an infectious RNA encoding the Ebola protein or
5 proteins of interest (see Figure 2A, 2B and 2C). The
6 replicon constructs include Ebola virus GP (SEQ ID
7 NO:1) cloned into a VEE replicon (VRepEboGP), Ebola
8 virus NP (SEQ ID NO:2) cloned into a VEE replicon
9 (VRepEboNP), Ebola virus VP24 (SEQ ID NO:3) cloned
10 into a VEE replicon (VRepEboVP24), Ebola virus VP30
11 (SEQ ID NO:4) or VP30#2 (SEQ ID NO:7) cloned into a
12 VEE replicon (VRepEboVP30 or VRepEboVP30(#2)), Ebola
13 virus VP35 (SEQ ID NO:5) cloned into a VEE replicon
14 (VRepEboVP35), and Ebola virus VP40 (SEQ ID NO:6)
15 cloned into a VEE replicon (VRepEboVP40). The
16 replicon DNA or RNA can be used as a vaccine for
17 inducing protection against infection with Ebola.
18 Use of helper RNAs containing sequences necessary for
19 packaging of the viral replicon transcripts will
20 result in the production of virus-like particles
21 containing replicon RNAs (Figure 3). These packaged
22 replicons will infect host cells and initiate a single
23 round of replication resulting in the expression of
24 the Ebola proteins in said infected cells. The
25 packaged replicon constructs (i.e. VEE virus replicon
26 particles, VRP) include those that express Ebola virus
27 GP (EboGPVRP), Ebola virus NP (EboNPVRP), Ebola virus
28 VP24 (EboVP24VRP), Ebola virus VP30 (EboVP30VRP or
29 EboVP30VRP(#2)), Ebola virus VP35 (EboVP35VRP), and
30 Ebola virus VP40 (EboVP40VRP).

31 In another embodiment, the present invention
32 relates to RNA molecules resulting from the
33 transcription of the constructs described above. The
34 RNA molecules can be prepared by *in vitro* transcription
35 using methods known in the art and described in the
36 Examples below. Alternatively, the RNA molecules can be
37 produced by transcription of the constructs *in vivo*, and
38 isolating the RNA. These and other methods for

1 obtaining RNA transcripts of the constructs are known in
2 the art. Please see Current Protocols in Molecular
3 Biology. Frederick M. Ausubel et al. (eds.), John Wiley
4 and Sons, Inc. The RNA molecules can be used, for
5 example, as a direct RNA vaccine, or to transfect cells
6 along with RNA from helper plasmids, one of which
7 expresses VEE glycoproteins and the other VEE capsid
8 proteins, as described above, in order to obtain
9 replicon particles.

10 In a further embodiment, the present invention
11 relates to a method of producing the recombinant or
12 fusion protein which includes culturing the above-
13 described host cells under conditions such that the
14 DNA fragment is expressed and the recombinant or
15 fusion protein is produced thereby. The recombinant or
16 fusion protein can then be isolated using methodology
17 well known in the art. The recombinant or fusion
18 protein can be used as a vaccine for immunity against
19 infection with Ebola or as a diagnostic tool for
20 detection of Ebola infection.

21 In another embodiment, the present invention
22 relates to antibodies specific for the above-described
23 recombinant proteins (or polypeptides). For instance,
24 an antibody can be raised against a peptide having the
25 amino acid sequence of any of SEQ ID NO:17-25, or
26 against a portion thereof of at least 10 amino acids,
27 preferably, 11-15 amino acids. Persons with ordinary
28 skill in the art using standard methodology can raise
29 monoclonal and polyclonal antibodies to the protein(or
30 polypeptide) of the present invention, or a unique
31 portion thereof. Materials and methods for producing
32 antibodies are well known in the art (see for example
33 Goding, In Monoclonal Antibodies: Principles and
34 Practice, Chapter 4, 1986).

35 In a further embodiment, the present invention
36 relates to a method of detecting the presence of
37 antibodies against Ebola virus in a sample. Using

1 standard methodology well known in the art, a
2 diagnostic assay can be constructed by coating on a
3 surface (i.e. a solid support for example, a
4 microtitration plate, a membrane (e.g. nitrocellulose
5 membrane) or a dipstick), all or a unique portion of
6 any of the Ebola proteins described above or any
7 combination thereof, and contacting it with the serum
8 of a person or animal suspected of having Ebola. The
9 presence of a resulting complex formed between the
10 Ebola protein(s) and serum antibodies specific
11 therefor can be detected by any of the known methods
12 common in the art, such as fluorescent antibody
13 spectroscopy or colorimetry. This method of detection
14 can be used, for example, for the diagnosis of Ebola
15 infection and for determining the degree to which an
16 individual has developed virus-specific Abs after
17 administration of a vaccine.

18 In yet another embodiment, the present invention
19 relates to a method for detecting the presence of
20 Ebola virion proteins in a sample. Antibodies against
21 GP, NP, and the VP proteins could be used for
22 diagnostic assays. Using standard methodology well
23 known in the art, a diagnostics assay can be
24 constructed by coating on a surface (i.e. a solid
25 support, for example, a microtitration plate or a
26 membrane (e.g. nitrocellulose membrane)), antibodies
27 specific for any of the Ebola proteins described
28 above, and contacting it with serum or a tissue sample
29 of a person suspected of having Ebola infection. The
30 presence of a resulting complex formed between the
31 protein or proteins in the serum and antibodies
32 specific therefor can be detected by any of the known
33 methods common in the art, such as fluorescent
34 antibody spectroscopy or colorimetry. This method of
35 detection can be used, for example, for the diagnosis
36 of Ebola virus infection.

37 In another embodiment, the present invention
38 relates to a diagnostic kit which contains any

1 combination of the Ebola proteins described above and
2 ancillary reagents that are well known in the art and
3 that are suitable for use in detecting the presence of
4 antibodies to Ebola in serum or a tissue sample.
5 Tissue samples contemplated can be from monkeys,
6 humans, or other mammals.

7 In yet another embodiment, the present invention
8 relates to DNA or nucleotide sequences for use in
9 detecting the presence of Ebola virus using the
10 reverse transcription-polymerase chain reaction (RT-
11 PCR). The DNA sequence of the present invention can
12 be used to design primers which specifically bind to
13 the viral RNA for the purpose of detecting the
14 presence of Ebola virus or for measuring the amount
15 of Ebola virus in a sample. The primers can be any
16 length ranging from 7 to 400 nucleotides, preferably
17 at least 10 to 15 nucleotides, or more preferably 18
18 to 40 nucleotides. Reagents and controls necessary
19 for PCR reactions are well known in the art. The
20 amplified products can then be analyzed for the
21 presence of viral sequences, for example by gel
22 fractionation, with or without hybridization, by
23 radiochemistry, and immunochemistry techniques.

24 In yet another embodiment, the present invention
25 relates to a diagnostic kit which contains PCR primers
26 specific for Ebola virus and ancillary reagents for
27 use in detecting the presence or absence of Ebola in a
28 sample using PCR. Samples contemplated can be obtained
29 from human, animal, e.g., horse, donkey, pig, mouse,
30 hamster, monkey, or other mammals, birds, and insects,
31 such as mosquitoes.

32 In another embodiment, the present invention
33 relates to an Ebola vaccine comprising VRPs that
34 express one or more of the Ebola proteins described
35 above. The vaccine is administered to a subject
36 wherein the replicon is able to initiate one round of
37 replication producing the Ebola proteins to which a

1 protective immune response is initiated in said
2 subject.

3 It is likely that the protection afforded by
4 these genes is due to both the humoral (antibodies
5 (Abs)) and cellular (cytotoxic T cells (CTLs)) arms of
6 the immune system. Protective immunity induced to a
7 specific protein may comprise humoral immunity,
8 cellular immunity, or both. The only Ebola virus
9 protein known to be on the outside of the virion is
10 the GP. The presence of GP on the virion surface
11 makes it a likely target for GP-specific Abs that may
12 bind either extracellular virions or infected cells
13 expressing GP on their surfaces. Serum transfer
14 studies in this invention demonstrate that Abs that
15 recognize GP protect mice against lethal Ebola virus
16 challenge.

17 In contrast, transfer of Abs specific for NP,
18 VP24, VP30, VP35, or VP40 did not protect mice against
19 lethal Ebola challenge. This data, together with the
20 fact that these are internal virion proteins that are
21 not readily accessible to Abs on either extracellular
22 virions or the surface of infected cells, suggest that
23 the protection induced in mice by these proteins is
24 mediated by CTLs.

25 CTLs can bind to and lyse virally infected cells.
26 This process begins when the proteins produced by
27 cells are routinely digested into peptides. Some of
28 these peptides are bound by the class I or class II
29 molecules of the major histocompatibility complex
30 (MHC), which are then transported to the cell surface.
31 During virus infections, viral proteins produced
32 within infected cells also undergo this process. CTLs
33 that have receptors that bind to both a specific
34 peptide and the MHC molecule holding the peptide lyse
35 the peptide-bearing cell, thereby limiting virus
36 replication. Thus, CTLs are characterized as being
37 specific for a particular peptide and restricted to a
38 class I or class II MHC molecule.

1 CTLs may be induced against any of the Ebola
2 virus proteins, as all of the viral proteins are
3 produced and digested within the infected cell. Thus,
4 protection to Ebola virus could involve CTLs against
5 GP, NP, VP24, VP30, VP35, and/or VP40. It is
6 especially noteworthy that the VP proteins varied in
7 their protective efficacy when tested in genetically
8 inbred mice that differ at the MHC locus. This,
9 together with the inability to demonstrate a role for
10 Abs in protection induced by the VP proteins, strongly
11 supports a role for CTLs. These data also suggest
12 that an eventual vaccine candidate may include several
13 Ebola virus proteins, or several CTL epitopes, capable
14 of inducing broad protection in outbred populations
15 (e.g. people). We have identified two sequences
16 recognized by CTLs. They are Ebola virus NP SEQ ID
17 NO:24 and Ebola virus VP24 SEQ ID NO:25. Testing is
18 in progress to identify the role of CTLs in protection
19 induced by each of these Ebola virus proteins and to
20 define the minimal sequence requirements for the
21 protective response. The CTL assay is well known in
22 the art.

23 An eventual vaccine candidate might
24 comprise these CTL sequences and others. These might
25 be delivered as synthetic peptides, or as fusion
26 proteins, alone or co-administered with cytokines
27 and/or adjuvants or carriers safe for human use, e.g.
28 aluminum hydroxide, to increase immunogenicity. In
29 addition, sequences such as ubiquitin can be added to
30 increase antigen processing for more effective CTL
31 responses.

32 In yet another embodiment, the present invention
33 relates to a method for providing immunity against
34 Ebola virus, said method comprising administering one
35 or more VRPs expressing any combination of the GP, NP,
36 VP24, VP30 or VP30#2, VP35 and VP40 Ebola proteins to
37 a subject such that a protective immune reaction is
38 generated.

1 Vaccine formulations of the present invention
2 comprise an immunogenic amount of a VRP, such as for
3 example EboVP24VRP described above, or, for a
4 multivalent vaccine, a combination of replicons, in a
5 pharmaceutically acceptable carrier. An "immunogenic
6 amount" is an amount of the VRP(s) sufficient to evoke
7 an immune response in the subject to which the vaccine
8 is administered. An amount of from about 10^4 - 10^8
9 focus-forming units per dose is suitable, depending
10 upon the age and species of the subject being treated.
11 The subject may be inoculated 2-3 times. Exemplary
12 pharmaceutically acceptable carriers include, but are
13 not limited to, sterile pyrogen-free water and sterile
14 pyrogen-free physiological saline solution.

15 Administration of the VRPs disclosed herein may
16 be carried out by any suitable means, including
17 parenteral injection (such as intraperitoneal,
18 subcutaneous, or intramuscular injection), in ovo
19 injection of birds, orally, or by topical application
20 of the virus (typically carried in a pharmaceutical
21 formulation) to an airway surface. Topical application
22 of the virus to an airway surface can be carried out
23 by intranasal administration (e.g., by use of dropper,
24 swab, or inhaler which deposits a pharmaceutical
25 formulation intranasally). Topical application of the
26 virus to an airway surface can also be carried out by
27 inhalation administration, such as by creating
28 respirable particles of a pharmaceutical formulation
29 (including both solid particles and liquid particles)
30 containing the replicon as an aerosol suspension, and
31 then causing the subject to inhale the respirable
32 particles. Methods and apparatus for administering
33 respirable particles of pharmaceutical formulations
34 are well known, and any conventional technique can be
35 employed. Oral administration may be in the form of
36 an ingestible liquid or solid formulation.

1 When the replicon RNA or DNA is used as a vaccine,
2 the replicon RNA or DNA can be administered directly
3 using techniques such as delivery on gold beads (gene
4 gun), delivery by liposomes, or direct injection, among
5 other methods known to people in the art. Any one or
6 more DNA constructs or replicating RNA described above
7 can be use in any combination effective to elicit an
8 immunogenic response in a subject. Generally, the
9 nucleic acid vaccine administered may be in an amount of
10 about 1-5 ug of nucleic acid per dose and will depend on
11 the subject to be treated, capacity of the subject's
12 immune system to develop the desired immune response,
13 and the degree of protection desired. Precise amounts
14 of the vaccine to be administered may depend on the
15 judgement of the practitioner and may be peculiar to
16 each subject and antigen.

17 The vaccine may be given in a single dose
18 schedule, or preferably a multiple dose schedule in
19 which a primary course of vaccination may be with 1-10
20 separate doses, followed by other doses given at
21 subsequent time intervals required to maintain and or
22 reinforce the immune response, for example, at 1-4
23 months for a second dose, and if needed, a subsequent
24 dose(s) after several months. Examples of suitable
25 immunization schedules include: (i) 0, 1 months and 6
26 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1
27 month, (iv) 0 and 6 months, or other schedules
28 sufficient to elicit the desired immune responses
29 expected to confer protective immunity, or reduce
30 disease symptoms, or reduce severity of disease.

31 The following examples are included to demonstrate
32 preferred embodiments of the invention. It should be
33 appreciated by those of skill in the art that the
34 techniques disclosed in the examples which follow
35 represent techniques discovered by the inventors and
36 thought to function well in the practice of the
37 invention, and thus can be considered to constitute
38 preferred modes for its practice. However, those of

1 skill in the art should, in light of the present
2 disclosure, appreciate that many changes can be made in
3 the specific embodiments which are disclosed and still
4 obtain a like or similar result without departing from
5 the spirit and scope of the invention.

6

7 The following MATERIALS AND METHODS were used in
8 the examples that follow.

9 Cells lines and viruses

10 BHK (ATCC CCL 10), Vero 76 (ATCC CRL 1587), and
11 Vero E6 (ATCC CRL 1586) cell lines were maintained in
12 minimal essential medium with Earle's salts, 5-10%
13 fetal bovine serum, and 50 µg/mL gentamicin sulfate.

14 For CTL assays, EL4 (ATCC TIB39), L5178Y (ATCC CRL
15 1723) and P815 (ATCC TIB64) were maintained in
16 Dulbecco's minimal essential medium supplemented with
17 5-10% fetal bovine serum and antibiotics.

18 A stock of the Zaire strain of Ebola virus
19 originally isolated from a patient in the 1976
20 outbreak (Mayinga) and passaged intracerebrally 3
21 times in suckling mice and 2 times in Vero cells was
22 adapted to adult mice through serial passage in
23 progressively older suckling mice (Bray et al., (1998)
24 *J. Infect. Dis.* **178**, 651-661). A plaque-purified
25 ninth-mouse-passage isolate which was uniformly lethal
26 for adult mice ("mouse-adapted virus") was propagated
27 in Vero E6 cells, aliquotted, and used in all mouse
28 challenge experiments and neutralization assays.

29 A stock of the Zaire strain of Ebola 1976 virus
30 was passaged spleen to spleen in strain 13 guinea pigs
31 four times. This guinea pig-adapted strain was used
32 to challenge guinea pigs.

33 Construction and packaging of recombinant VEE
34 virus replicons (VRPs)

35 Replicon RNAs were packaged into VRPs as
36 described (Pushko et al., 1997, supra). Briefly,
37 capped replicon RNAs were produced *in vitro* by T7 run-

1 off transcription of NotI-digested plasmid templates
2 using the RiboMAX T7 RNA polymerase kit (Promega).
3 BHK cells were co-transfected with the replicon RNAs
4 and the 2 helper RNAs expressing the structural
5 proteins of the VEE virus. The cell culture
6 supernatants were harvested approximately 30 hours
7 after transfection and the replicon particles were
8 concentrated and purified by centrifugation through a
9 20% sucrose cushion. The pellets containing the
10 packaged replicon particles were suspended in PBS and
11 the titers were determined by infecting Vero cells
12 with serial dilutions of the replicon particles and
13 enumerating the infected cells by indirect
14 immunofluorescence with antibodies specific for the
15 Ebola proteins.

16 Immunoprecipitation of Ebola virus proteins
17 expressed from VEE virus replicons

18 BHK cells were transfected with either the Ebola
19 virus GP, NP, VP24, VP30, VP35, or VP40 replicon RNAs.
20 At 24 h post-transfection, the culture medium was
21 replaced with minimal medium lacking cysteine and
22 methionine, and proteins were labeled for 1 h with
23 ³⁵S-labeled methionine and cysteine. Cell lysates or
24 supernatants (supe) were collected and
25 immunoprecipitated with polyclonal rabbit anti-Ebola
26 virus serum bound to protein A beads. ³⁵S-labeled
27 Ebola virus structural proteins from virions grown in
28 Vero E6 cells were also immunoprecipitated as a
29 control for each of the virion proteins.
30 Immunoprecipitated proteins were resolved by
31 electrophoresis on an 11% SDS-polyacrylamide gel and
32 were visualized by autoradiography.

33 Vaccination of Mice With VEE Virus Replicons

34 Groups of 10 BALB/c or C57BL/6 mice per experiment
35 were subcutaneously injected at the base of the neck
36 with 2×10^6 focus-forming units of VRPs encoding the
37 Ebola virus genes. As controls, mice were also

1 injected with either a control VRP encoding the Lassa
2 nucleoprotein (NP) or with PBS. For booster
3 inoculations, animals received identical injections at
4 1 month intervals. Data are recorded as the combined
5 results of 2 or 3 separate experiments.

6 Ebola Infection of Mice

7 One month after the final booster inoculation,
8 mice were transferred to a BSL-4 containment area and
9 challenged by intraperitoneal (ip) inoculation of 10
10 plaque-forming units (pfu) of mouse-adapted Ebola
11 virus (approximately 300 times the dose lethal for 50%
12 of adult mice). The mice were observed daily, and
13 morbidity and mortality were recorded. Animals
14 surviving at day 21 post-infection were injected again
15 with the same dose of virus and observed for another
16 21 days.

17 In some experiments, 4 or 5 mice from vaccinated
18 and control groups were anesthetized and exsanguinated
19 on day 4 (BALB/c mice) or day 5 (C57BL/6 mice)
20 following the initial viral challenge. The viral
21 titers in individual sera were determined by plaque
22 assay.

23 Passive Transfer Of Immune Sera to Naive Mice.

24 Donor sera were obtained 28 days after the third
25 inoculation with 2×10^6 focus-forming units of VRPs
26 encoding the indicated Ebola virus gene, the control
27 Lassa NP gene, or from unvaccinated control mice. One
28 mL of pooled donor sera was administered
29 intraperitoneally (ip) to naive, syngeneic mice 24 h
30 prior to intraperitoneal challenge with 10 pfu of
31 mouse-adapted Ebola virus.

32 Vaccination and Challenge of Guinea Pigs.

33 EboGPVRP or EboNPVRP (1×10^7 focus-forming units
34 in 0.5ml PBS) were administered subcutaneously to
35 inbred strain 2 or strain 13 guinea pigs (300-400g).
36 Groups of five guinea pigs were inoculated on days 0
37 and 28 at one (strain 2) or two (strain 13) dorsal

1 sites. Strain 13 guinea pigs were also boosted on day
2 126. One group of Strain 13 guinea pigs was
3 vaccinated with both the GP and NP constructs. Blood
4 samples were obtained after vaccination and after
5 viral challenge. Guinea pigs were challenged on day
6 56 (strain 2) or day 160 (strain 13) by subcutaneous
7 administration of 1000 LD₅₀ (1×10^4 PFU) of guinea
8 pig-adapted Ebola virus. Animals were observed daily
9 for 60 days, and morbidity (determined as changes in
10 behavior, appearance, and weight) and survival were
11 recorded. Blood samples were taken on the days
12 indicated after challenge and viremia levels were
13 determined by plaque assay.

14 Virus titration and neutralization assay. Viral
15 stocks were serially diluted in growth medium,
16 adsorbed onto confluent Vero E6 cells in 6- or 12-well
17 dishes, incubated for 1 hour at 37°C, and covered with
18 an agarose overlay (Moe, J. et al. (1981) *J. Clin.*
19 *Microbiol.* 13:791-793). A second overlay containing 5%
20 neutral red solution in PBS or agarose was added 6
21 days later, and plaques were counted the following
22 day. Pooled pre-challenge serum samples from some of
23 the immunized groups were tested for the presence of
24 Ebola-neutralizing antibodies by plaque reduction
25 neutralization assay. Aliquots of Ebola virus in
26 growth medium were mixed with serial dilutions of test
27 serum, or with normal serum, or medium only, incubated
28 at 37°C for 1 h, and used to infect Vero E6 cells.
29 Plaques were counted 1 week later.

30 Cytotoxic T cell assays. BALB/c and C57BL/6 mice
31 were inoculated with VRPs encoding Ebola virus NP or
32 VP24 or the control Lassa NP protein. Mice were
33 euthanized at various times after the last inoculation
34 and their spleens removed. The spleens were gently
35 ruptured to generate single cell suspensions. Spleen
36 cells (1×10^6 / ml) were cultured *in vitro* for 2 days
37 in the presence of 10-25 μ M of peptides synthesized

1 from Ebola virus NP or VP24 amino acid sequences, and
2 then for an additional 5 days in the presence of
3 peptide and 10% supernatant from concanavalin A-
4 stimulated syngeneic spleen cells. Synthetic peptides
5 were made from Ebola virus amino acid sequences
6 predicted by a computer algorithm (HLA Peptide Binding
7 Predictions, Parker, K. C., et al. (1994) *J. Immunol.*
8 **152**:163) to have a likelihood of meeting the MHC
9 class I binding requirements of the BALB/c (H-2^d) and
10 C57BL/6 (H-2^b) haplotypes. Only 2 of 8 peptides
11 predicted by the algorithm and tested to date have
12 been identified as containing CTL epitopes. After *in*
13 *vitro* restimulation, the spleen cells were tested in a
14 standard ⁵¹chromium-release assay well known in the
15 art (see, for example, Hart et al. (1991) *Proc. Natl.*
16 *Acad. Sci. USA* **88**: 9449-9452). Percent specific lysis
17 of peptide-coated, MHC-matched or mismatched target
18 cells was calculated as:

19

$$\frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100$$

21

22
23 Spontaneous cpm are the number of counts
24 released from target cells incubated in medium.
25 Maximum cpm are obtained by lysing target cells with
26 1% Triton X-100. Experimental cpm are the counts from
27 wells in which target cells are incubated with varying
28 numbers of effector (CTL) cells. Target cells tested
29 were L5178Y lymphoma or P815 mastocytoma cells (MHC
30 matched to the H2^d BALB/c mice and EL4 lymphoma cells
31 (MHC matched to the H2^b C57BL/6 mice). The
32 effector:target (E:T) ratios tested were 25:1, 12:1,
33 6:1 and 3:1.

34

EXAMPLE 1

35 Survival Of Mice Inoculated With VRPs Encoding
36 Ebola Proteins. Mice were inoculated two or three
37 times at 1 month intervals with 2×10^6 focus-forming

1 units of VRPs encoding individual Ebola virus genes,
 2 or Lassa virus NP as a control, or with phosphate
 3 buffered saline (PBS). Mice were challenged with 10
 4 pfu of mouse-adapted Ebola virus one month after the
 5 final immunization. The mice were observed daily, and
 6 morbidity and mortality data are shown in Table 1A for
 7 BALB/c mice and Table 1B for C57BL/6 mice. The viral
 8 titers in individual sera of some mice on day 4
 9 (BALB/c mice) or day 5 (C57BL/6 mice) following the
 10 initial viral challenge were determined by plaque
 11 assay.

12
 13 **Table 1.** Survival Of Mice Inoculated With VRPs
 14 Encoding Ebola Proteins

15 **A. BALB/c Mice**

16	VRP	#Injections	S/T ¹ (%)	MDD ²	V/T ³	Viremia ⁴
17	EboNP	3	30/30 (100%)	5/5	5.2	
18		2	19/20 (95%)	7	5/5	4.6
19						
20	EboGP	3	15/29 (52%)	8	1/5	6.6
21		2	14/20 (70%)	7	3/5	3.1
22						
23	EboVP24	3	27/30 (90%)	8	5/5	5.2
24		2	19/20 (95%)	6	4/4	4.8
25						
26	EboVP30	3	17/20 (85%)	7	5/5	6.2
27		2	11/20 (55%)	7	5/5	6.5
28						
29	EboVP35	3	5/19 (26%)	7	5/5	6.9
30		2	4/20 (20%)	7	5/5	6.5
31						
32	EboVP40	3	14/20 (70%)	8	5/5	4.6
33		2	17/20 (85%)	7	5/5	5.6
34						
35	LassaNP	3	0/29 (0%)	7	5/5	8.0
36		2	0/20 (0%)	7	5/5	8.4

37

1	none (PBS)	3	1/30 (3%)	6	5/5	8.3
2		2	0/20 (0%)	6	5/5	8.7

3

4 **B. C57BL/6 Mice**

5

6	VRP	#Injections	S/T ¹ (%)	MDD ²	V/T ³	Viremia ⁴
7						
8	EboNP	3	15/20 (75%)	8	5/5	4.1
9		2	8/10 (80%)	9	ND ⁵	ND
10						
11	EboGP	3	19/20 (95%)	10	0/5	--
12		2	10/10 (100%)	-	ND	ND
13						
14	EboVP24	3	0/20 (0%)	7	5/5	8.6
15						
16	EboVP30	3	2/20 (10%)	8	5/5	7.7
17						
18	EboVP35	3	14/20 (70%)	8	5/5	4.5
19						
20	EboVP40	3	1/20 (5%)	7	4/4	7.8
21						
22	LassaNP	3	1/20 (5%)	7	4/4	8.6
23		2	0/10 (0%)	7	ND	ND
24						
25	none (PBS)	3	3/20 (15%)	7	5/5	8.6
26		2	0/10 (0%)	7	ND	ND

27

28 ¹S/T, Survivors/total challenged.29 ²MDD, Mean day to death30 ³V/T, Number of mice with viremia/total number tested.

31 ⁴Geometric mean of Log₁₀ viremia titers in PFU/mL. Standard
 32 errors for all groups were 1.5 or less, except for the group of
 33 BALB/c mice given 2 inoculations of EboGP, which was 2.2.

34 ⁵ND, not determined.

35

36

37

EXAMPLE 2**VP24-Immunized BALB/c Mice Survive A High-Dose
Challenge With Ebola virus.**

BALB/c mice were inoculated two times with 2×10^6 focus-forming units of EboVP24VRP. Mice were challenged with either 1×10^3 pfu or 1×10^5 pfu of mouse-adapted Ebola virus 1 month after the second inoculation. Morbidity and mortality data for these mice are shown in Table 2.

Table 2. VP24-Immunized BALB/c Mice Survive A High-Dose Challenge With Ebola virus

<u>Replicon</u>	<u>Challenge Dose</u>	<u>Survivors/Total</u>
EboVP24	1×10^3 pfu (3×10^4 LD ₅₀)	5/5
EboVP24	1×10^5 pfu (3×10^6 LD ₅₀)	5/5
None	1×10^3 pfu (3×10^4 LD ₅₀)	0/4
None	1×10^5 pfu (3×10^6 LD ₅₀)	0/3

EXAMPLE 3**Passive Transfer Of Immune Sera Can Protect
Naive Mice From A Lethal Challenge Of Ebola Virus.**

Donor sera were obtained 28 days after the third inoculation with 2×10^6 focus-forming units of VRPs encoding the indicated Ebola virus gene, the control Lassa NP gene, or from unvaccinated control mice. One mL of pooled donor sera was administered

1 intraperitoneally (ip) to naive, syngeneic mice 24 h
2 prior to intraperitoneal challenge with 10 pfu of
3 mouse-adapted Ebola virus.
4

5 **Table 3.** Passive Transfer of Immune Sera Can Protect
6 Unvaccinated Mice from a Lethal Challenge of Ebola
7 Virus
8

9 A. BALB/c Mice

10 Specificity of	Survivors	Mean Day
11 <u>Donor Sera</u>	<u>/Total</u>	<u>of Death</u>
12 Ebola GP	15/20	8
13 Ebola NP	1/20	7
14 Ebola VP24	0/20	6
15 Ebola VP30	0/20	7
16 Ebola VP35	ND ¹	ND
17 Ebola VP40	0/20	6
18 Lassa NP	0/20	7
19 Normal mouse sera	0/20	6

20
21 B. C57BL/6 Mice

22 Specificity of	Survivors	Mean Day
23 <u>Donor Sera</u>	<u>/Total</u>	<u>of Death</u>
24 Ebola GP	17/20	7
25 Ebola NP	0/20	7
26 Ebola VP24	ND	ND
27 Ebola VP30	ND	ND
28 Ebola VP35	0/20	7
29 Ebola VP40	ND	ND
30 Lassa NP	0/20	7
31 Normal mouse sera	0/20	7

32
33 ¹ND, not determined
34
35
36
37

EXAMPLE 4**Immunogenicity and Efficacy of VRepEboGP and VRepEboNP in Guinea Pigs.**

EboGPVRP or EboNPVRP (1×10^7 IU in 0.5ml PBS) were administered subcutaneously to inbred strain 2 or strain 13 guinea pigs (300-400g). Groups of five guinea pigs were inoculated on days 0 and 28 at one (strain 2) or two (strain 13) dorsal sites. Strain 13 guinea pigs were also boosted on day 126. One group of Strain 13 guinea pigs was vaccinated with both the GP and NP constructs. Blood samples were obtained after vaccination and after viral challenge.

Sera from vaccinated animals were assayed for antibodies to Ebola by plaque-reduction neutralization, and ELISA. Vaccination with VRepEboGP or NP induced high titers of antibodies to the Ebola proteins (Table 4) in both guinea pig strains. Neutralizing antibody responses were only detected in animals vaccinated with the GP construct (Table 4).

Guinea pigs were challenged on day 56 (strain 2) or day 160 (strain 13) by subcutaneous administration of 1000 LD₅₀ (10^4 PFU) of guinea pig-adapted Ebola virus. Animals were observed daily for 60 days, and morbidity (determined as changes in behavior, appearance, and weight) and survival were recorded. Blood samples were taken on the days indicated after challenge and viremia levels were determined by plaque assay. Strain 13 guinea pigs vaccinated with the GP construct, alone or in combination with NP, survived lethal Ebola challenge (Table 4). Likewise, vaccination of strain 2 inbred guinea pigs with the GP construct protected 3/5 animals against death from lethal Ebola challenge, and significantly prolonged the mean day of death (MDD) in one of the two animals that died (Table 4). Vaccination with NP alone did not protect either guinea pig strain.

Table 4. Immunogenicity and efficacy of VRepEboGP and VRepEboNP in guinea pigs
A. Strain 2 guinea pigs

VRP	ELISA ^a	PRNT ₅₀	Survivors/ total (MDD ^b)		Viremia ^c d7 d14	
GP	4.1	30	3/5	(13+2.8)	2.3	1.8
NP	3.9	<10	0/5	(9.2+1.1)	3.0	--
Mock	<1.5	<10	0/5	(8.8+0.5)	3.9	--

B. Strain 13 guinea pigs

VRP	ELISA ^a	PRNT ₅₀	Survivors/ total (MDD ^b)		Viremia ^c d7 d14	
GP	4.0	140	5/5		<2.0	<2.0
GP/NP	3.8	70	5/5		<2.0	<2.0
NP	2.8	<10	1/5	(8.3+2.2)	4.6	--
Lassa NP	<1.5	<10	2/5	(8.3+0.6)	4.8	--

^aData are expressed as geometric mean titers, log₁₀.

^bMDD, mean day to death

^cGeometric mean of log₁₀ viremia titers in PFU/mL. Standard errors for all groups were 0.9 or less.

EXAMPLE 5

Induction of murine CTL responses to Ebola virus NP and Ebola virus VP24 proteins.

BALB/c and C57BL/6 mice were inoculated with VRPs encoding Ebola virus NP or VP24. Mice were euthanized at various times after the last inoculation and their spleens removed. Spleen cells (1×10^6 /ml) were cultured *in vitro* for 2 days in the presence of 10 to 25 μ M of peptides, and then for an additional 5 days in the presence of peptide and 10% supernatant from concanavalin A-stimulated syngeneic spleen cells. After *in vitro* restimulation, the spleen cells were tested in a standard ⁵¹chromium-release assay. Percent specific lysis of peptide-coated, MHC-matched or mismatched target cells was calculated as:

1

2 Experimental cpm- Spontaneous cpm x 100

3 Maximum cpm-Spontaneous cpm

4

5 In the experiments shown, spontaneous release did not
6 exceed 15%.

7

8 **Table 5.** Induction of murine CTL responses to Ebola
9 virus NP and Ebola virus VP24 proteins.

		% Specific Lysis	
		E:T ratio	
<u>Mice, VRP¹</u>	<u>Peptide²</u>	<u>Cell³</u>	<u>25</u>
BALB/c, VP24	None	P815	55
BALB/c, VP24	SEQ ID NO:25	P815	93
C57BL/6, EboNP	None	EL4	2
C57BL/6, EboNP ⁴	SEQ ID NO:24	EL4	70
C57BL/6, EboNP	Lassa NP	EL4	2
C57BL/6, LassaNP	None	L5178Y	1
C57BL/6, LassaNP	SEQ ID NO:24	L5178Y	0
C57BL/6, LassaNP	None	EL4	2
C57BL/6, LassaNP	SEQ ID NO:24	EL4	6

22 ¹ Indicates the mouse strain used and the VRP used as the *in*
23 *vivo* immunogen. *In vitro* restimulation was performed using SEQ
24 ID NO:24 peptide for BALB/c mice and SEQ ID NO:23 for all
25 C57BL/6 mice shown.

26 ² Indicates the peptide used to coat the target cells for the
27 chromium release assay.

28 ³ Target cells are MHC-matched to the effector cells, except
29 for the L5178Y cells that are C57BL/6 mismatched.

30 ⁴ High levels of specific lysis (>40%) were also observed using
31 E:T ratios of 12, 6, 3, or 1:1.

32 RESULTS AND DISCUSSION

33 Ebola Zaire 1976 (Mayinga) virus causes acute
34 hemorrhagic fever characterized by high mortality.
35 There are no current vaccines or effective therapeutic
36 measures to protect individuals who are exposed to
37 this virus. In addition, it is not known which genes

1 are essential for evoking protective immunity and
2 should therefore be included in a vaccine designed for
3 human use. In this study, the GP, NP, VP24, VP30,
4 VP35, and VP40 virion protein genes of the Ebola Zaire
5 1976 (Mayinga) virus were cloned and inserted into a
6 Venezuelan equine encephalitis (VEE) virus replicon
7 vector (VRep) as shown in Figure 2A and 2B. These
8 VReps were packaged as VEE replicon particles (VRPs)
9 using the VEE virus structural proteins provided as
10 helper RNAs, as shown in Figure 3. This enables
11 expression of the Ebola virus proteins in host cells.
12 The Ebola virus proteins produced from these
13 constructs were characterized *in vitro* and were shown
14 to react with polyclonal rabbit anti-Ebola virus
15 antibodies bound to Protein A beads following SDS gel
16 electrophoresis of immunoprecipitated proteins (Figure
17 4).

18 The Ebola virus genes were sequenced from the VEE
19 replicon clones and are listed here as SEQ ID NO:1
20 (GP), 2 (NP), 3 (VP24), 4 (VP30), 5 (VP35), 6 (VP40),
21 and 7 (VP30#2) as described below. The corresponding
22 amino acid sequences of the Ebola proteins expressed
23 from these replicons are listed as SEQ ID NO: 17, 18,
24 19, 20, 21, 22, and 23, respectively. Changes in the
25 DNA sequence relative to the sequence published by
26 Sanchez *et al.* (1993) are described relative to the
27 nucleotide (nt) sequence number from GenBank
28 (accession number L11365).

29 The sequence we obtained for Ebola virus GP (SEQ
30 ID NO:1) differed from the GenBank sequence by a
31 transition from A to G at nt 8023. This resulted in a
32 change in the amino acid sequence from Ile to Val at
33 position 662 (SEQ ID NO: 17).

34 The DNA sequence we obtained for Ebola virus NP
35 (SEQ ID NO:2) differed from the GenBank sequence at
36 the following 4 positions: insertion of a C residue
37 between nt 973 and 974, deletion of a G residue at nt
38 979, transition from C to T at nt 1307, and a

1 transversion from A to C at nt 2745. These changes
2 resulted in a change in the protein sequence from Arg
3 to Glu at position 170 and a change from Leu to Phe at
4 position 280 (SEQ ID NO: 18).

5 The Ebola virus VP24 (SEQ ID NO:3) gene differed
6 from the GenBank sequence at 6 positions, resulting in
7 3 nonconservative changes in the amino acid sequence.
8 The changes in the DNA sequence of VP24 consisted of a
9 transversion from G to C at nt 10795, a transversion
10 from C to G at nt 10796, a transversion from T to A at
11 nt 10846, a transversion from A to T at nt 10847, a
12 transversion from C to G at nt 11040, and a
13 transversion from C to G at nt 11041. The changes in
14 the amino acid sequence of VP24 consisted of a Cys to
15 Ser change at position 151, a Leu to His change at
16 position 168, and a Pro to Gly change at position 233
17 (SEQ ID NO: 19).

18 We have included 2 different sequences for the
19 Ebola virus VP30 gene (SEQ ID NOS:4 and SEQ ID NO:7).
20 Both of these sequences differ from the GenBank
21 sequence by the insertion of an A residue in the
22 upstream noncoding sequence between nt 8469 and 8470
23 and an insertion of a T residue between nt 9275 and
24 9276 that results in a change in the open reading
25 frame of VP30 and VP30#2 after position 255 (SEQ ID
26 NOS:20 and SEQ ID NO:23). As a result, the C-terminus
27 of the VP30 protein differs significantly from that
28 previously reported. In addition to these 2 changes,
29 the VP30#2 gene in SEQ ID NO:23 contains a
30 conservative transition from T to C at nt 9217.
31 Because the primers originally used to clone the VP30
32 gene into the replicon were designed based on the
33 GenBank sequence, the first clone that we constructed
34 (SEQ ID NO:4) did not contain what we believe to be
35 the authentic C-terminus of the protein. Therefore,
36 in the absence of the VP30 stop codon, the C-terminal
37 codon was replaced with 37 amino acids derived from
38 the vector sequence. The resulting VP30 construct

1 therefore differed from the GenBank sequence in that
2 it contained 32 amino acids of VP30 sequence
3 (positions 256 to 287, SEQ ID NO:20) and 37 amino
4 acids of irrelevant sequence (positions 288 to 324,
5 SEQ ID NO:20) in the place of the C-terminal 5 amino
6 acids reported in GenBank. However, inclusion of 37
7 amino acids of vector sequence in place of the C-
8 terminal amino acid (Pro, SEQ ID NO:23) did not
9 inhibit the ability of the protein to serve as a
10 protective antigen in BALB/c mice. We are currently
11 examining the ability of the new VEE replicon
12 construct (SEQ ID NO:7), which we believe contains the
13 authentic C-terminus of VP30 (VP30#2, SEQ ID NO:23),
14 to protect mice against a lethal Ebola challenge.

15 The DNA sequence for Ebola virus VP35 (SEQ ID
16 NO:5) differed from the GenBank sequence by a
17 transition from T to C at nt 4006, a transition from T
18 to C at nt 4025, and an insertion of a T residue
19 between nt 4102 and 4103. These sequence changes
20 resulted in a change from a Ser to a Pro at position
21 293 and a change from Phe to Ser at position 299 (SEQ
22 ID NO:21). The insertion of the T residue resulted in
23 a change in the open reading frame of VP35 from that
24 previously reported by Sanchez et al. (1993) following
25 amino acid number 324. As a result, Ebola virus VP35
26 encodes for a protein of 340 amino acids, where amino
27 acids 325 to 340 (SEQ ID NO:21) differ from and
28 replace the C-terminal 27 amino acids of the
29 previously published sequence.

30 Sequencing of VP30 and VP35 was also performed
31 on RT/PCR products from RNA derived from cells that
32 were infected with Ebola virus 1976, Ebola virus 1995
33 or the mouse-adapted Ebola virus. The changes noted
34 above for the VRep constructs were also found in these
35 Ebola viruses. Thus, we believe that these changes are
36 real events and not artifacts of cloning.

37 The Ebola virus VP40 differed from the GenBank
38 sequence by a transversion from a C to G at nt 4451

1 and a transition from a G to A at nt 5081. These
2 sequence changes did not alter the protein sequence of
3 VP40 (SEQ ID NO:22) from that of the published
4 sequence.

5 To evaluate the protective efficacy of
6 individual Ebola virus proteins and to determine
7 whether the major histocompatibility (MHC) genes
8 influence the immune response to Ebola virus antigens,
9 two MHC-incompatible strains of mice were vaccinated
10 with VRPs expressing an Ebola protein. As controls for
11 these experiments, some mice were injected with VRPs
12 expressing the nucleoprotein of Lassa virus or were
13 injected with phosphate-buffered saline (PBS).
14 Following Ebola virus challenge, the mice were
15 monitored for morbidity and mortality, and the results
16 are shown in Table 1.

17 The GP, NP, VP24, VP30, and VP40 proteins of
18 Ebola virus generated either full or partial
19 protection in BALB/c mice, and may therefore be
20 beneficial components of a vaccine designed for human
21 use. Vaccination with VRPs encoding the NP protein
22 afforded the best protection. In this case, 100% of
23 the mice were protected after three inoculations and
24 95% of the mice were protected after two inoculations.
25 The VRP encoding VP24 also protected 90% to 95% of
26 BALB/c mice against Ebola virus challenge. In separate
27 experiments (Table 2), two or three inoculations with
28 VRPs encoding the VP24 protein protected BALB/c mice
29 from a high dose (1×10^5 plaque-forming units ($3 \times$
30 10^6 LD50)) of mouse-adapted Ebola virus.

31 Vaccination with VRPs encoding GP protected 52-
32 70% of BALB/c mice. The lack of protection was not
33 due to a failure to respond to the VRP encoding GP, as
34 all mice had detectable Ebola virus-specific serum
35 antibodies after vaccination.

36 Some protective efficacy was also observed in
37 BALB/c mice vaccinated two or three times with VRPs
38 expressing the VP30 protein (55% and 85%,

1 respectively), or the VP40 protein (70% and 80%,
2 respectively). The VP35 protein was not efficacious
3 in the BALB/c mouse model, as only 20% and 26% of the
4 mice were protected after either two or three doses,
5 respectively.

6 Geometric mean titers of viremia were markedly
7 reduced in BALB/c mice vaccinated with VRPs encoding
8 Ebola virus proteins after challenge with Ebola virus,
9 indicating an ability of the induced immune responses
10 to reduce virus replication (Table 1A). In this study,
11 immune responses to the GP protein were able to clear
12 the virus to undetectable levels within 4 days after
13 challenge in some mice.

14 When the same replicons were examined for their
15 ability to protect C57BL/6 mice from a lethal
16 challenge of Ebola virus, only the GP, NP, and VP35
17 proteins were efficacious (Table 1B). The best
18 protection, 95% to 100%, was observed in C57BL/6 mice
19 inoculated with VRPs encoding the GP protein.
20 Vaccination with VRPs expressing NP protected 75% to
21 80% of the mice from lethal disease. In contrast to
22 what was observed in the BALB/c mice, the VP35 protein
23 was the only VP protein able to significantly protect
24 the C57BL/6 mice. In this case, 3 inoculations with
25 VRPs encoding VP35 protected 70% of the mice from
26 Ebola virus challenge. The reason behind the
27 differences in protection in the two mouse strains is
28 not known but is believed to be due to the ability of
29 the immunogens to sufficiently stimulate the cellular
30 immune system. As with the BALB/c mice, the effects
31 of the induced immune responses were also observed in
32 reduced viremias and, occasionally, in a prolonged
33 time to death of C57BL/6 mice.

34 VRPs expressing Ebola virus GP or NP were also
35 evaluated for protective efficacy in a guinea pig
36 model. Sera from vaccinated animals were assayed for
37 antibodies to Ebola by western blotting, IFA, plaque-
38 reduction neutralization, and ELISA. Vaccination with

1 either VRP (GP or NP) induced high titers of
2 antibodies to the Ebola proteins (Table 4) in both
3 guinea pig strains. Neutralizing antibody responses
4 were only detected in animals vaccinated with the VRP
5 expressing GP (Table 4).

6 Vaccination of strain 2 inbred guinea pigs with
7 the GP construct protected 3/5 animals against death
8 from lethal Ebola challenge, and significantly
9 prolonged the mean day of death in one of the two
10 animals that died (Table 4). All of the strain 13
11 guinea pigs vaccinated with the GP construct, alone or
12 in combination with NP, survived lethal Ebola
13 challenge (Table 4). Vaccination with NP alone did not
14 protect either guinea pig strain from challenge with
15 the guinea pig-adapted Ebola virus.

16 To identify the immune mechanisms that mediate
17 protection against Ebola virus and to determine
18 whether antibodies are sufficient to protect against
19 lethal disease, passive transfer studies were
20 performed. One mL of immune sera, obtained from mice
21 previously vaccinated with one of the Ebola virus
22 VRPs, was passively administered to unvaccinated mice
23 24 hours before challenge with a lethal dose of mouse-
24 adapted Ebola virus. Antibodies to GP, but not to NP
25 or the VP proteins, protected mice from an Ebola virus
26 challenge (Table 3). Antibodies to GP protected 75% of
27 the BALB/c mice and 85% of the C57BL/6 mice from
28 death. When the donor sera were examined for their
29 ability to neutralize Ebola virus in a plaque-
30 reduction neutralization assay, a 1:20 to 1:40
31 dilution of the GP-specific antisera reduced the
32 number of viral plaque-forming units by at least 50%
33 (data not shown). In contrast, antisera to the NP and
34 VP proteins did not neutralize Ebola virus at a 1:20
35 or 1:40 dilution. These results are consistent with
36 the finding that GP is the only viral protein found on
37 the surface of Ebola virus, and is likely to induce
38 virus-neutralizing antibodies.

1 Since the NP and VP proteins of Ebola virus are
2 internal virion proteins to which antibodies are not
3 sufficient for protection, it is likely that cytotoxic
4 T lymphocytes (CTLs) are also important for protection
5 against Ebola virus. Initial studies aimed at
6 identifying cellular immune responses to individual
7 Ebola virus proteins expressed from VRPs identified
8 CTL responses to the VP24 and NP proteins (Table 5).
9 One CTL epitope that we identified for the Ebola virus
10 NP is recognized by C57BL/6 (H-2^b) mice, and has an
11 amino acid sequence of, or contained within, the
12 following 11 amino acids: VYQVNNLEEIC (SEQ ID NO:24).
13 Vaccination with EboNPVRP and *in vitro* restimulation
14 of spleen cells with this peptide consistently induces
15 strong CTL responses in C57BL/6 (H-2^b) mice. *In vivo*
16 vaccination to Ebola virus NP is required to detect
17 the CTL activity, as evidenced by the failure of cells
18 from C57BL/6 mice vaccinated with Lassa NP to develop
19 lytic activity to peptide (SEQ ID NO:24) after *in*
20 *vitro* restimulation with it. Specific lysis has been
21 observed using very low effector:target ratios (<2:1).
22 This CTL epitope is H-2^b restricted in that it is not
23 recognized by BALB/c (H-2^d) cells treated the same way
24 (data not shown), and H-2^b effector cells will not
25 lyse MHC-mismatched target cells coated with this
26 peptide.

27 A CTL epitope in the VP24 protein was also
28 identified. It is recognized by BALB/c (H-2^d) mice,
29 and has an amino acid sequence of, or contained
30 within, the following 23 amino acids:
31 LKFINKLDALLVVNYNGLLSSIF (SEQ ID NO:25). In the data
32 shown in Table 5, high (>90%) specific lysis of P815
33 target cells coated with this peptide was observed.
34 The background lysis of cells that were not peptide-
35 coated was also high (>50%), which is probably due to
36 the activity of natural killer cells. We are planning
37 to repeat this experiment using the L5178Y target

1 cells, which are not susceptible to natural killer
2 cells.

3 Future studies will focus on determining the
4 fine specificities of these CTL responses and the
5 essential amino acids that constitute these CTL
6 epitopes. Additional studies to identify other CTL
7 epitopes on Ebola virus GP, NP, VP24, VP30, VP35, and
8 VP40 will be performed. To evaluate the role of these
9 CTLs in protection against Ebola virus, lymphocytes
10 will be restimulated *in vitro* with peptides containing
11 the CTL epitopes, and adoptively transferred into
12 unvaccinated mice prior to Ebola virus challenge. In
13 addition, future studies will examine the CTL
14 responses to the other Ebola virus proteins to better
15 define the roles of the cell mediated immune responses
16 involved in protection against Ebola virus infection.

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1

2 What is claimed is:

3 1. A DNA fragment which encodes a GP Ebola protein,
4 said DNA fragment comprising the sequence specified in
5 SEQ ID NO:1, or a polynucleotide fragment comprising
6 at least 15 nucleotides.

7

8 2. A DNA fragment which encodes a NP Ebola protein,
9 said DNA fragment comprising the sequence specified in
10 SEQ ID NO:2, or a polynucleotide fragment comprising
11 at least 15 nucleotides.

12

13 3. A DNA fragment which encodes a VP24 Ebola protein,
14 said DNA fragment comprising the sequence specified in
15 SEQ ID NO:3, or a polynucleotide fragment comprising
16 at least 15 nucleotides.

17

18 4. A DNA fragment which encodes a VP30 Ebola protein,
19 said DNA fragment comprising the sequence specified in
20 any of SEQ ID NO:4 and SEQ ID NO:7, or a
21 polynucleotide fragment comprising at least 15
22 nucleotides.

23

24 5. A DNA fragment which encodes a VP35 Ebola protein,
25 said DNA fragment comprising the sequence specified in
26 SEQ ID NO:5, or a polynucleotide fragment comprising
27 at least 15 nucleotides.

28

29 6. A DNA fragment which encodes a VP40 Ebola protein,
30 said DNA fragment comprising the sequence specified in
31 SEQ ID NO:6, or a polynucleotide fragment comprising
32 at least 15 nucleotides.

33

34 7. A DNA fragment which encodes a GP Ebola protein
35 said DNA fragment comprising a DNA sequence encoding
36 at least 5 amino acids specified in SEQ ID NO:17 or a
37 conservative substitution thereof.

- 1
- 2 8. A DNA fragment which encodes a NP Ebola protein
- 3 said DNA fragment comprising a DNA sequence encoding
- 4 at least 5 amino acids specified in SEQ ID NO:18 or a
- 5 conservative substitution thereof.
- 6
- 7 9. A DNA fragment which encodes a VP24 Ebola protein
- 8 said DNA fragment comprising a DNA sequence encoding
- 9 at least 5 amino acids specified in SEQ ID NO:19 or a
- 10 conservative substitution thereof.
- 11
- 12 10. A DNA fragment which encodes a VP30 Ebola protein
- 13 said DNA fragment comprising a DNA sequence encoding
- 14 at least 5 amino acids specified in any of SEQ ID
- 15 NO:20 and SEQ ID NO:23 or a conservative substitution
- 16 thereof.
- 17
- 18 11. A DNA fragment which encodes a VP35 Ebola protein
- 19 said DNA fragment comprising a DNA sequence encoding
- 20 at least 5 amino acids specified in SEQ ID NO:21 or a
- 21 conservative substitution thereof.
- 22
- 23 12. A DNA fragment which encodes a VP40 Ebola protein
- 24 said DNA fragment comprising a DNA sequence encoding
- 25 at least 5 amino acids specified in SEQ ID NO:22 or a
- 26 conservative substitution thereof.
- 27
- 28 13. A recombinant DNA construct comprising:
- 29 (i) a vector, and
- 30 (ii) at least one of the Ebola virus DNA
- 31 fragments chosen from the group consisting of SEQ ID
- 32 NO:1, 2, 3, 4, 5, 6 and 7 or a fragment thereof
- 33 comprising at least 15 nucleotides.
- 34
- 35 14. A recombinant DNA construct comprising:
- 36 (i) a vector, and
- 37 (ii) at least one of the Ebola virus DNA
- 38 fragments chosen from the group consisting of SEQ ID

1 NO: 17, 18, 19, 20, 21, 22, 23, 24 and 25 or a
2 conservative substitution thereof.

3

4 15. The recombinant DNA construct of claim 13 wherein
5 said DNA fragment induces a cytotoxic T lymphocyte
6 response or antibody response.

7

8 16. The recombinant DNA construct of claim 14 wherein
9 said DNA fragment induces a cytotoxic T lymphocyte
10 response or antibody response.

11

12 17. A recombinant DNA construct according to claim 13
13 wherein said vector is an expression vector.

14

15 18. A recombinant DNA construct according to claim 13
16 wherein said vector is a prokaryotic vector.

17

18 19. A recombinant DNA construct according to claim 13
19 wherein said vector is a eukaryotic vector.

20

21 20. A recombinant DNA construct according to claim 14
22 wherein said vector is an expression vector.

23

24 21. A recombinant DNA construct according to claim 14
25 wherein said vector is a prokaryotic vector.

26

27 22. A recombinant DNA construct according to claim 14
28 wherein said vector is a eukaryotic vector.

29

30 23. The recombinant DNA construct of claim 17 wherein
31 said vector is a VEE virus replicon vector.

32

33 24. The recombinant DNA construct of claim 20 wherein
34 said vector is a VEE virus replicon vector.

35

36 25. The recombinant DNA construct according to claim
37 23 wherein said Ebola virus DNA fragments are from
38 Ebola Zaire 1976.

- 1
2 26. The recombinant DNA construct according to claim
3 25 wherein said construct is VRepEboVP24.
4
5 27. The recombinant DNA construct according to claim
6 25 wherein said construct is VRepEboVP30.
7
8 28. The recombinant DNA construct according to claim
9 25 wherein said construct is VRepEboVP35.
10
11 29. The recombinant DNA construct according to claim
12 25 wherein said construct is VRepEboVP40.
13
14 30. The recombinant DNA construct according to claim
15 25 wherein said construct is for VRepEboNP.
16
17 31. The recombinant DNA construct according to claim
18 25 wherein said construct is for VRepEboGP.
19
20 32. The recombinant DNA construct according to claim
21 25 wherein said construct is for VRepEboVP30(#2).
22
23 33. Self replicating RNA produced from a construct
24 chosen from the group consisting of EboVP24ReP,
25 EboVP30ReP, EboVP35ReP, EboVP40ReP, EboVPNPreP,
26 EboVPGPreP, and EboVP30ReP(#2).
27
28 34. Infectious alphavirus particles produced from
29 packaging the self replicating RNA of claim 33.
30
31 35. A pharmaceutical composition comprising infectious
32 alphavirus particles according to claim 34 in an
33 effective immunogenic amount in a pharmaceutically
34 acceptable carrier and/or adjuvant.
35
36 36. A host cell transformed with a recombinant DNA
37 construct according to claim 13.

1

2 37. A host cell transformed with a recombinant DNA
3 construct according to claim 14.

4

5 38. A host cell according to claim 36 wherein said
6 host cell is prokaryotic.

7

8 39. A host cell according to claim 36 wherein said
9 host cell is eukaryotic.

10

11 40. A host cell according to claim 37 wherein said
12 host cell is prokaryotic.

13

14 41. A host cell according to claim 37 wherein said
15 host cell is eukaryotic.

16

17 42. A method for producing Ebola virus proteins
18 comprising culturing the cells according to claim 36
19 under conditions such that said DNA fragment is
20 expressed and said Ebola protein is produced.

21

22 43. A method for producing Ebola virus proteins
23 comprising culturing the cells according to claim 37
24 under conditions such that said DNA fragment is
25 expressed and said Ebola protein is produced.

26

27 44. A method for producing Ebola virus proteins
28 comprising culturing the cells according to claim 38
29 under conditions such that said DNA fragment is
30 expressed and said Ebola protein is produced.

31

32 45. A method for producing Ebola virus proteins
33 comprising culturing the cells according to claim 39
34 under conditions such that said DNA fragment is
35 expressed and said Ebola protein is produced.

36

37

- 1 46. An isolated and purified Ebola GP protein
2 specified in SEQ ID NO:17 and conservative
3 substitutions thereof, or an immunologically
4 identifiable portion thereof.
5
- 6 47. An isolated and purified Ebola NP protein
7 specified in SEQ ID NO:18 and conservative
8 substitutions thereof or an immunologically
9 identifiable portion thereof.
10
- 11 48. An isolated and purified Ebola VP24 protein
12 specified in SEQ ID NO:19 and conservative
13 substitutions thereof or an immunologically
14 identifiable portion thereof.
15
- 16 49. An isolated and purified Ebola VP30 protein
17 specified in any of SEQ ID NO:20 and SEQ ID NO:23 and
18 conservative substitutions thereof or an
19 immunologically identifiable portion thereof.
20
- 21 50. An isolated and purified Ebola VP35 protein
22 specified in SEQ ID NO:21 and conservative
23 substitutions thereof or an immunologically
24 identifiable portion thereof.
25
- 26 51. An isolated and purified Ebola VP40 protein
27 specified in SEQ ID NO:22 and conservative
28 substitutions thereof or an immunologically
29 identifiable portion thereof.
30
- 31 52. An antibody to a peptide encoded by the sequence
32 specified in SEQ ID NO:17, 18, 19, 20, 21, 22, 23, 24,
33 and 25.
34
- 35 53. A method for detecting Ebola virus infection
36 comprising contacting a sample from a subject
37 suspected of having Ebola virus infection with a
38 antibody according to claim 52 and detecting the

1 presence or absence by detecting the presence or
2 absence of a complex formed between the Ebola protein
3 and antibodies specific therefor.
4

5 54. A method for detecting the presence or absence of
6 Ebola virus GP RNA in a sample using the polymerase
7 chain reaction using primers for Ebola GP nucleic acid
8 sequence specified in SEQ ID NO:1 for GP.
9

10 55. An Ebola infection diagnostic kit comprising at
11 least 12 consecutive nucleotides of SEQ ID NO:1
12 specific for the amplification of DNA or RNA of Ebola
13 virus in a sample using the polymerase chain reaction
14 and ancillary reagents suitable for use in such a
15 reaction for detecting the presence or absence of
16 Ebola virus DNA or RNA in a sample.
17

18 56. A vaccine for Ebola comprising alphavirus
19 particles of claim 34.
20

21 57. A method for the diagnosis of Ebola virus
22 infection comprising the steps of:
23 (i) contacting a sample from an individual
24 suspected of having Ebola virus infection with an
25 antibody to Ebola proteins according to claim 52; and
26 (ii) detecting the presence or absence of Ebola
27 virus infection by detecting the presence or absence
28 of a complex formed between Ebola proteins and
29 antibodies specific therefor.
30

31 58. A pharmaceutical composition comprising the self
32 replicating RNA of claim 33 in an effective immunogenic
33 amount in a pharmaceutically acceptable carrier and/or
34 adjuvant.
35

36 59. A pharmaceutical composition comprising one or more
37 recombinant DNA constructs chosen from the group
38 consisting of VRepEboVP24, VRepEboVP30, VRepEboVP35,

1 VRepEboVP40, VRepEboNP, VRepEboGP, and VRepEboVP30(#2),
2 in a pharmaceutically acceptable amount, in a
3 pharmaceutically acceptable carrier and/or adjuvant.
4

5 60. A pharmaceutical composition comprising comprising a
6 peptide encoded by any of SEQ ID NO:24 and SEQ ID NO:25,
7 in a pharmaceutically acceptable amount, in a
8 pharmaceutically acceptable carrier and/or adjuvant.
9

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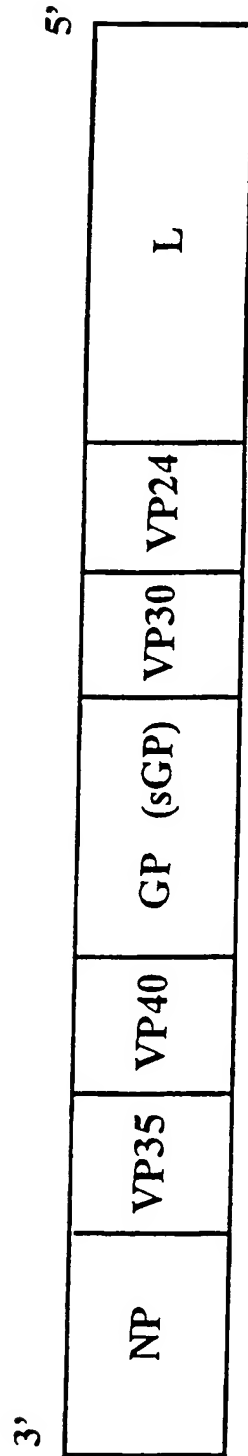
32

33

34

FIG.1

Organization of the Ebola Virus Genome



- NP Major Nucleocapsid Protein
- VP35 Phosphoprotein
- VP40 Membrane-Associated Matrix Protein
- GP Transmembrane Glycoprotein
- sGP Secreted Glycoprotein
- VP30 Ribonucleoprotein Associated (Minor)
- VP24 Membrane-Associated Protein (Minor)
- L RNA-Dependent RNA Polymerase

2 / 6

FIG.2A

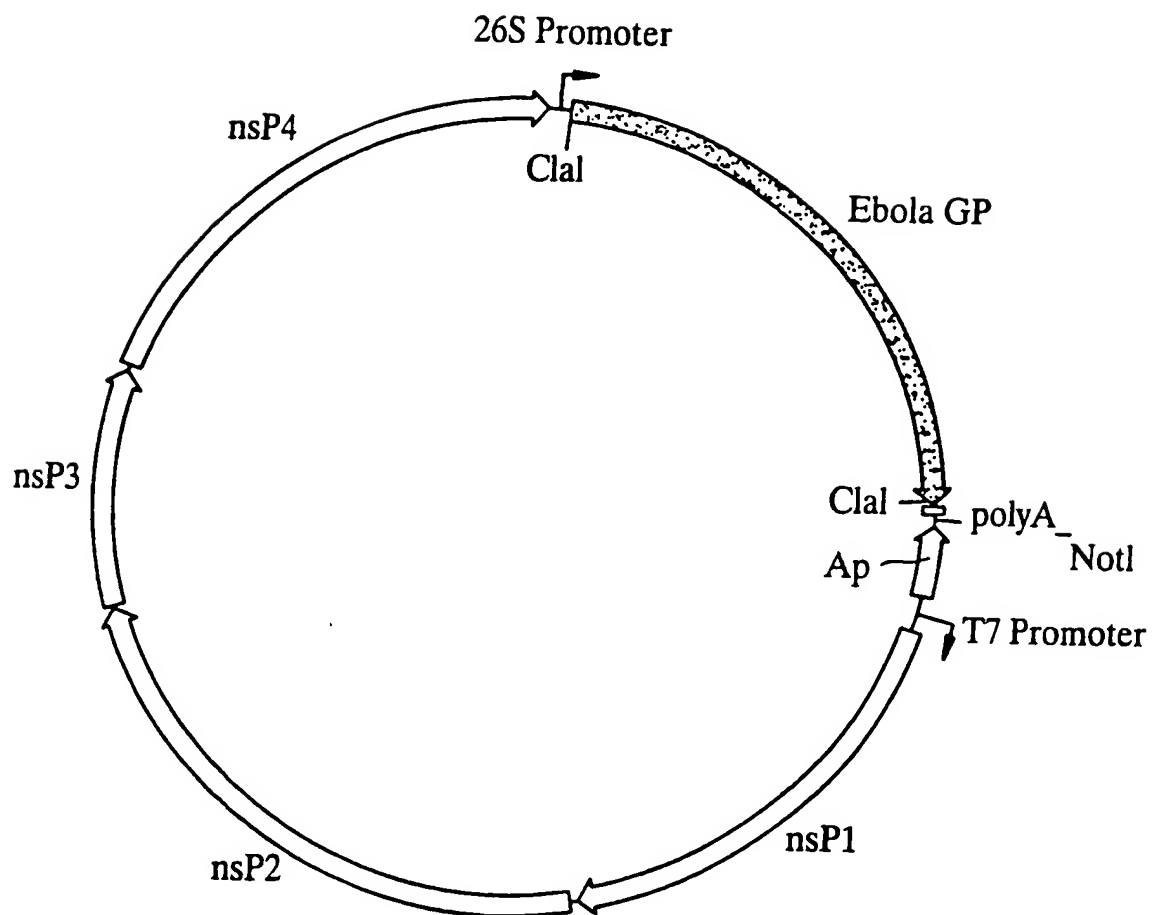


FIG.2B

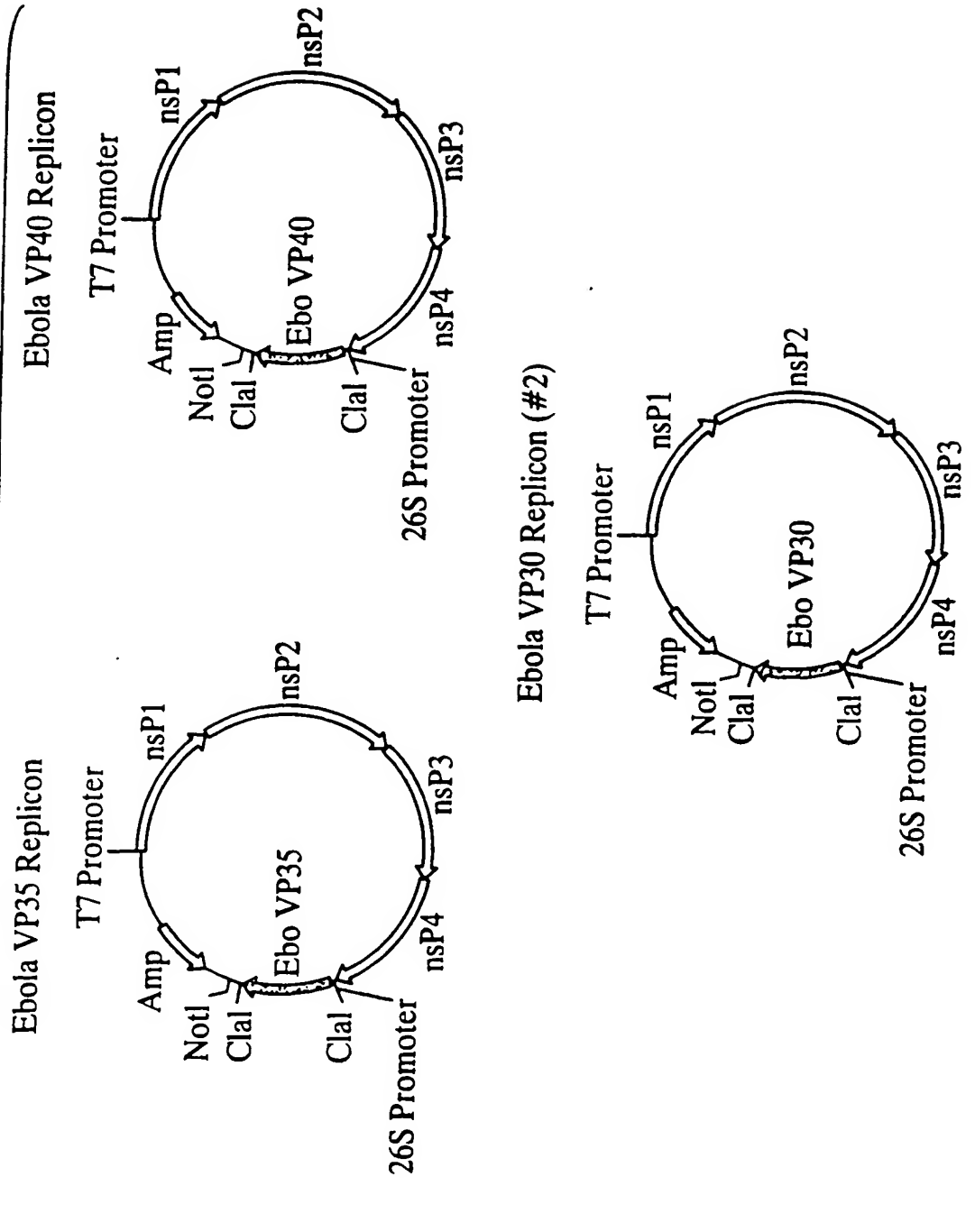


FIG.2C

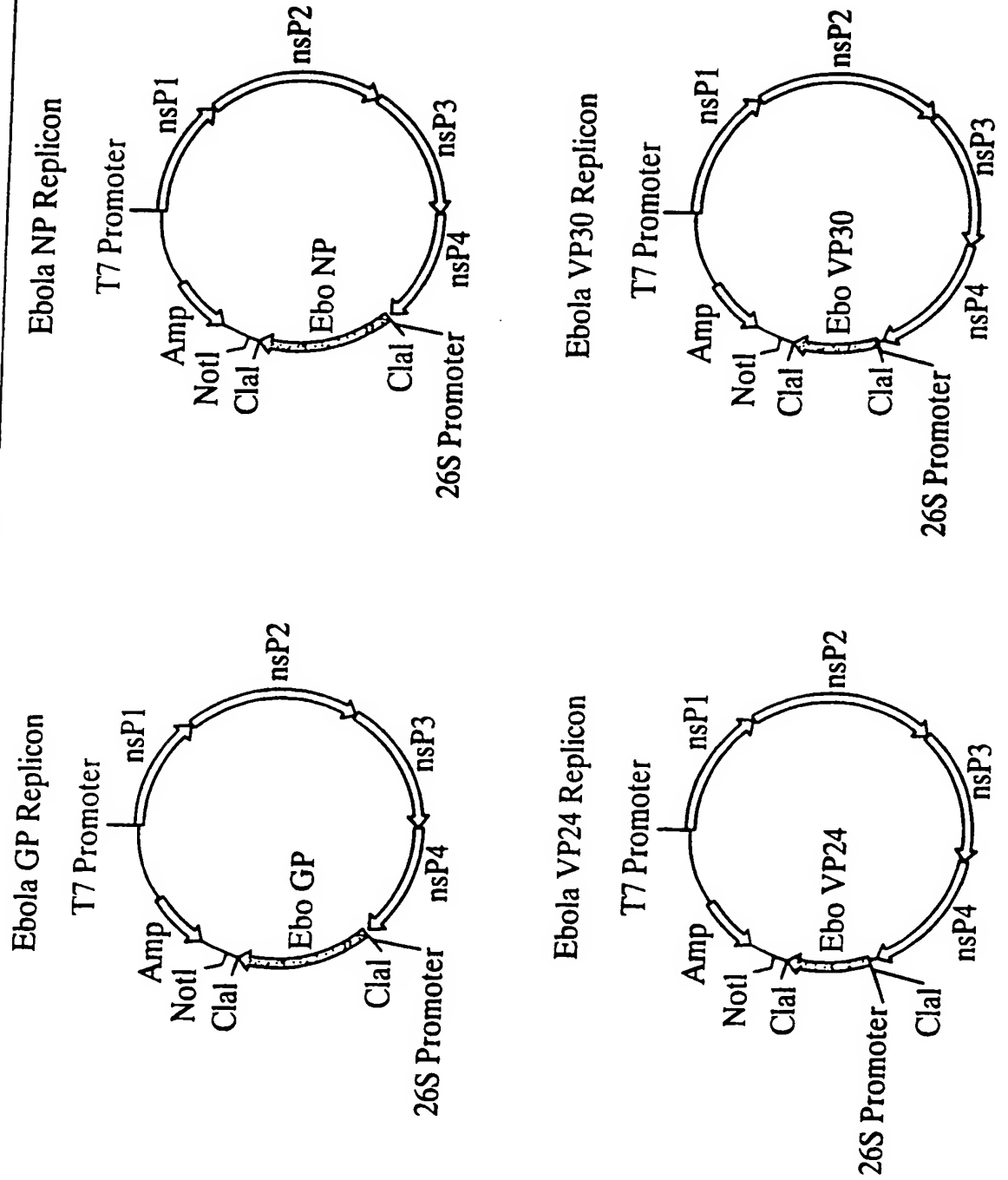


FIG.3

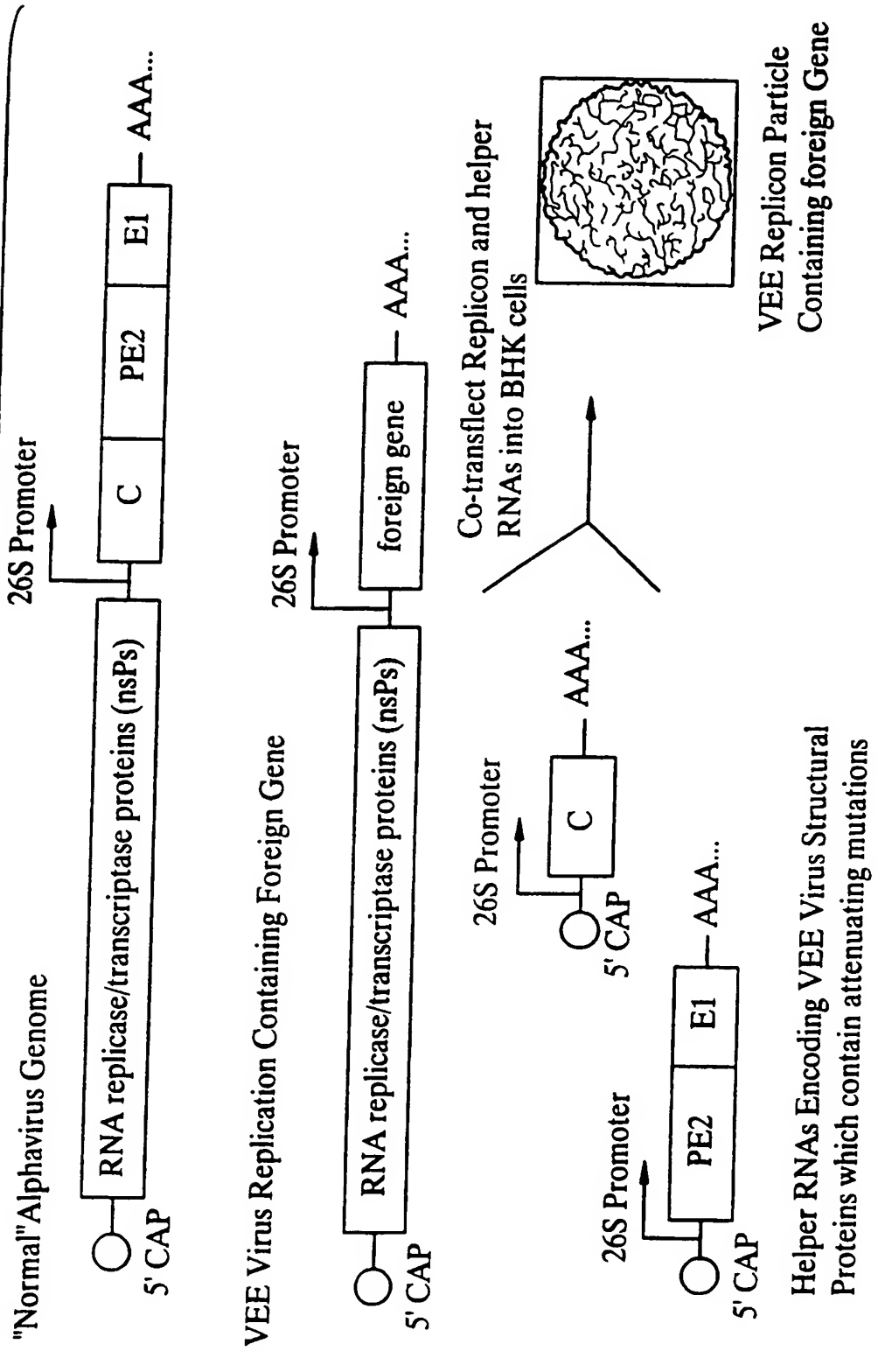
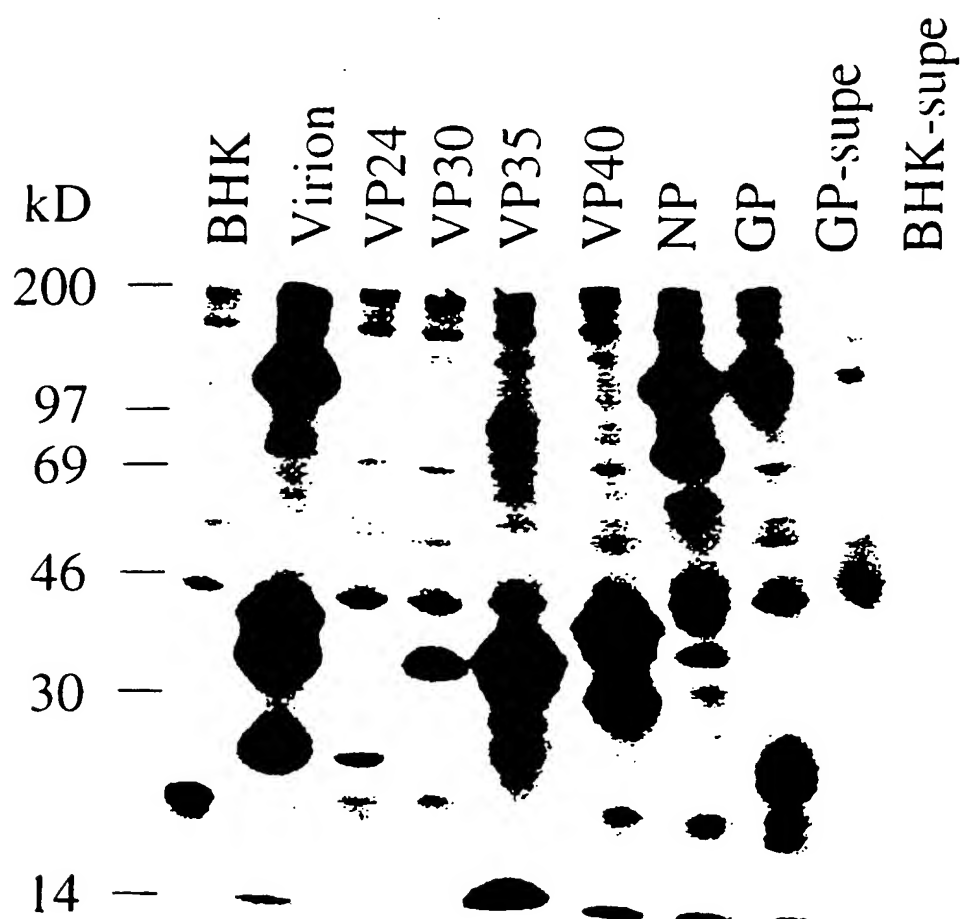


FIG. 4

Eboli Proteins Expressed from
VEE Replicons



SEQUENCE LISTING

<110> United States Army Medical Research Institute of
Infectious Diseases
Hart, Mary Katherine
Wilson, Julie A.
Pushko, Peter
Smith, Jonathan F.
Schmaljohn, Alan L.

<120> Ebola Virion Proteins Expressed from Venezuelan Equine
Encephalitis (VEE) Virus Replicons

<130> 003/141/SAP

<140> PCT/US99/14311

<141> 1999-06-22

<150> US 60/091,403

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<212> DNA

<213> Ebola Zaire

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ttgggtaatt	atccttttcc	aaagaacatt	ttccatccca	160
cttggagtca	tccacaatag	cacattacag	gttagtgatg	200
tcgacaaact	agttttgtcgt	gacaaactgt	catccacaaa	240
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gtggcaactg	acgtgccatc	tgcaactaaa	agatggggct	320
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<212> DNA

<213> Ebola Zaire

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<400> 3

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<211> 973

<212> DNA

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<400> 4

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<211> 1148

<212> DNA

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<220>

<400> 5

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<213> Ebola Zaire

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<400> 6

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<211> 36

<212> DNA

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<400> 17

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Tyr	Arg	Gly	Thr	Thr	Phe	Ala	Glu	Gly	Val
				175					180
Val	Ala	Phe	Leu	Ile	Leu	Pro	Gln	Ala	Lys
				185					190
Lys	Asp	Phe	Phe	Ser	Ser	His	Pro	Leu	Arg
				195					200
Glu	Pro	Val	Asn	Ala	Thr	Glu	Asp	Pro	Ser
				205					210
Ser	Gly	Tyr	Tyr	Ser	Thr	Thr	Ile	Arg	Tyr
				215					220
Gln	Ala	Thr	Gly	Phe	Gly	Thr	Asn	Glu	Thr
				225					230

Glu Tyr Leu Phe	Glu Val Asp Asn Leu Thr	235	240
Tyr Val Gln Leu	Glu Ser Arg Phe Thr Pro	245	250
Gln Phe Leu Leu	Gln Leu Asn Glu Thr Ile	255	260
Tyr Thr Ser Gly	Lys Arg Ser Asn Thr Thr	265	270
Gly Lys Leu Ile	Trp Lys Val Asn Pro Glu	275	280
Ile Asp Thr Thr	Ile Gly Glu Trp Ala Phe	285	290
Trp Glu Thr Lys	Lys Asn Leu Thr Arg Lys	295	300
Ile Arg Ser Glu	Glu Leu Ser Phe Thr Val	305	310
Val Ser Asn Gly	Ala Lys Asn Ile Ser Gly	315	320
Gln Ser Pro Ala	Arg Thr Ser Ser Asp Pro	325	330
Gly Thr Asn Thr	Thr Thr Glu Asp His Lys	335	340
Ile Met Ala Ser	Glu Asn Ser Ser Ala Met	345	350
Val Gln Val His	Ser Gln Gly Arg Glu Ala	355	360
Ala Val Ser His	Leu Thr Thr Leu Ala Thr	365	370
Ile Ser Thr Ser	Pro Gln Ser Leu Thr Thr	375	380
Lys Pro Gly Pro	Asp Asn Ser Thr His Asn	385	390
Thr Pro Val Tyr	Lys Leu Asp Ile Ser Glu	395	400
Ala Thr Gln Val	Glu Gln His His Arg Arg	405	410
Thr Asp Asn Asp	Ser Thr Ala Ser Asp Thr	415	420
Pro Ser Ala Thr	Thr Ala Ala Gly Pro Pro	425	430
Lys Ala Glu Asn	Thr Asn Thr Ser Lys Ser	435	440
Thr Asp Phe Leu	Asp Pro Ala Thr Thr Thr	445	450
Ser Pro Gln Asn	His Ser Glu Thr Ala Gly	455	460
Asn Asn Asn Thr	His His Gln Asp Thr Gly	465	470
Glu Glu Ser Ala	Ser Ser Gly Lys Leu Gly	475	480
Leu Ile Thr Asn	Thr Ile Ala Gly Val Ala	485	490
Gly Leu Ile Thr	Gly Gly Arg Arg Thr Arg	495	500

Arg	Glu	Ala	Ile	Val	Asn	Ala	Gln	Pro	Lys	
				505						510
Cys	Asn	Pro	Asn	Leu	His	Tyr	Trp	Thr	Thr	
				515						520
Gln	Asp	Glu	Gly	Ala	Ala	Ile	Gly	Leu	Ala	
				525						530
Trp	Ile	Pro	Tyr	Phe	Gly	Pro	Ala	Ala	Glu	
				535						540
Gly	Ile	Tyr	Ile	Glu	Gly	Leu	Met	His	Asn	
				545						550
Gln	Asp	Gly	Leu	Ile	Cys	Gly	Leu	Arg	Gln	
				555						560
Leu	Ala	Asn	Glu	Thr	Thr	Gln	Ala	Leu	Gln	
				565						570
Leu	Phe	Leu	Arg	Ala	Thr	Thr	Glu	Leu	Arg	
				575						580
Thr	Phe	Ser	Ile	Leu	Asn	Arg	Lys	Ala	Ile	
				585						590
Asp	Phe	Leu	Leu	Gln	Arg	Trp	Gly	Gly	Thr	
				595						600
Cys	His	Ile	Leu	Gly	Pro	Asp	Cys	Cys	Ile	
				605						610
Glu	Pro	His	Asp	Trp	Thr	Lys	Asn	Ile	Thr	
				615						620
Asp	Lys	Ile	Asp	Gln	Ile	Ile	His	Asp	Phe	
				625						630
Val	Asp	Lys	Thr	Leu	Pro	Asp	Gln	Gly	Asp	
				635						640
Asn	Asp	Asn	Trp	Trp	Thr	Gly	Trp	Arg	Gln	
				645						650
Trp	Ile	Pro	Ala	Gly	Ile	Gly	Val	Thr	Gly	
				655						660
Val	Val	Ile	Ala	Val	Ile	Ala	Leu	Phe	Cys	
				665						670
Ile	Cys	Lys	Phe	Val	Phe					
				675						

<210> 18

<211> 739

<212> PRT

<213> Ebola Zaire

<220>

<400> 18

Met	Asp	Ser	Arg	Pro	Gln	Lys	Ile	Trp	Met	
1				5					10	
Ala	Pro	Ser	Leu	Thr	Glu	Ser	Asp	Met	Asp	
				15					20	
Tyr	His	Lys	Ile	Leu	Thr	Ala	Gly	Leu	Ser	

	25		30
Val Gln Gln Gly	Ile Val Arg Gln Arg	Val	
	35		40
Ile Pro Val Tyr	Gln Val Asn Asn Leu	Glu	
	45		50
Glu Ile Cys Gln	Leu Ile Ile Gln Ala	Phe	
	55		60
Glu Ala Gly Val	Asp Phe Gln Glu Ser	Ala	
	65		70
Asp Ser Phe Leu	Leu Met Leu Cys Leu	His	
	75		80
His Ala Tyr Gln	Gly Asp Tyr Lys Leu	Phe	
	85		90
Leu Glu Ser Gly	Ala Val Lys Tyr Leu	Glu	
	95		100
Gly His Gly Phe	Arg Phe Glu Val Lys	Lys	
	105		110
Arg Asp Gly Val	Lys Arg Leu Glu Glu	Leu	
	115		120
Leu Pro Ala Val	Ser Ser Gly Lys Asn	Ile	
	125		130
Lys Arg Thr Leu	Ala Ala Met Pro Glu	Glu	
	135		140
Glu Thr Thr Glu	Ala Asn Ala Gly Gln	Phe	
	145		150
Leu Ser Phe Ala	Ser Leu Phe Leu Pro	Lys	
	155		160
Leu Val Val Gly	Glu Lys Ala Cys Leu	Glu	
	165		170
Lys Val Gln Arg	Gln Ile Gln Val His	Ala	
	175		180
Glu Gln Gly Leu	Ile Gln Tyr Pro Thr	Ala	
	185		190
Trp Gln Ser Val	Gly His Met Met Val	Ile	
	195		200
Phe Arg Leu Met	Arg Thr Asn Phe Leu	Ile	
	205		210
Lys Phe Leu Leu	Ile His Gln Gly Met	His	
	215		220
Met Val Ala Gly	His Asp Ala Asn Asp	Ala	
	225		230
Val Ile Ser Asn	Ser Val Ala Gln Ala	Arg	
	235		240
Phe Ser Gly Leu	Leu Ile Val Lys Thr	Val	
	245		250
Leu Asp His Ile	Leu Gln Lys Thr Glu	Arg	
	255		260
Gly Val Arg Leu	His Pro Leu Ala Arg	Thr	
	265		270
Ala Lys Val Lys	Asn Glu Val Asn Ser	Phe	
	275		280
Lys Ala Ala Leu	Ser Ser Leu Ala Lys	His	
	285		290
Gly Glu Tyr Ala	Pro Phe Ala Arg Leu	Leu	

	295	300
Asn Leu Ser Gly Val Asn Asn Leu Glu His		
	305	310
Gly Leu Phe Pro Gln Leu Ser Ala Ile Ala		
	315	320
Leu Gly Val Ala Thr Ala His Gly Ser Thr		
	325	330
Leu Ala Gly Val Asn Val Gly Glu Gln Tyr		
	335	340
Gln Gln Leu Arg Glu Ala Ala Thr Glu Ala		
	345	350
Glu Lys Gln Leu Gln Gln Tyr Ala Glu Ser		
	355	360
Arg Glu Leu Asp His Leu Gly Leu Asp Asp		
	365	370
Gln Glu Lys Lys Ile Leu Met Asn Phe His		
	375	380
Gln Lys Lys Asn Glu Ile Ser Phe Gln Gln		
	385	390
Thr Asn Ala Met Val Thr Leu Arg Lys Glu		
	395	400
Arg Leu Ala Lys Leu Thr Glu Ala Ile Thr		
	405	410
Ala Ala Ser Leu Pro Lys Thr Ser Gly His		
	415	420
Tyr Asp Asp Asp Asp Asp Ile Pro Phe Pro		
	425	430
Gly Pro Ile Asn Asp Asp Asp Asn Pro Gly		
	435	440
His Gln Asp Asp Asp Pro Thr Asp Ser Gln		
	445	450
Asp Thr Thr Ile Pro Asp Val Val Val Asp		
	455	460
Pro Asp Asp Gly Ser Tyr Gly Glu Tyr Gln		
	465	470
Ser Tyr Ser Glu Asn Gly Met Asn Ala Pro		
	475	480
Asp Asp Leu Val Leu Phe Asp Leu Asp Glu		
	485	490
Asp Asp Glu Asp Thr Lys Pro Val Pro Asn		
	495	500
Arg Ser Thr Lys Gly Gly Gln Gln Lys Asn		
	505	510
Ser Gln Lys Gly Gln His Ile Glu Gly Arg		
	515	520
Gln Thr Gln Ser Arg Pro Ile Gln Asn Val		
	525	530
Pro Gly Pro His Arg Thr Ile His His Ala		
	535	540
Ser Ala Pro Leu Thr Asp Asn Asp Arg Arg		
	545	550
Asn Glu Pro Ser Gly Ser Thr Ser Pro Arg		
	555	560
Met Leu Thr Pro Ile Asn Glu Glu Ala Asp		

	565		570
Pro Leu Asp Asp	Ala Asp Asp Glu Thr Ser		
	575		580
Ser Leu Pro Pro	Leu Glu Ser Asp Asp Glu		
	585		590
Glu Gln Asp Arg	Asp Gly Thr Ser Asn Arg		
	595		600
Thr Pro Thr Val	Ala Pro Pro Ala Pro Val		
	605		610
Tyr Arg Asp His	Ser Glu Lys Lys Glu Leu		
	615		620
Pro Gln Asp Glu	Gln Gln Asp Gln Asp His		
	625		630
Thr Gln Glu Ala	Arg Asn Gln Asp Ser Asp		
	635		640
Asn Thr Gln Ser	Glu His Ser Phe Glu Glu		
	645		650
Met Tyr Arg His	Ile Leu Arg Ser Gln Gly		
	655		660
Pro Phe Asp Ala	Val Leu Tyr Tyr His Met		
	665		670
Met Lys Asp Glu	Pro Val Val Phe Ser Thr		
	675		680
Ser Asp Gly Lys	Glu Tyr Thr Tyr Pro Asp		
	685		690
Ser Leu Glu Glu	Glu Tyr Pro Pro Trp Leu		
	695		700
Thr Glu Lys Glu	Ala Met Asn Glu Glu Asn		
	705		710
Arg Phe Val Thr	Leu Asp Gly Gln Gln Phe		
	715		720
Tyr Trp Pro Val	Met Asn His Lys Asn Lys		
	725		730
Phe Met Ala Ile	Leu Gln His His Gln		
	735		

<210> 19

<211> 251

<212> PRT

<213> Ebola Zaire

<220>

<400> 19

Met Ala Lys Ala Thr Gly Arg Tyr Asn Leu	
1	5
Ile Ser Pro Lys Lys Asp Leu Glu Lys Gly	10
	15
Val Val Leu Ser Asp Leu Cys Asn Phe Leu	20
	25
	30

Val	Ser	Gln	Thr	Ile	Gln	Gly	Trp	Lys	Val	
				35					40	
Tyr	Trp	Ala	Gly	Ile	Glu	Phe	Asp	Val	Thr	
				45					50	
His	Lys	Gly	Met	Ala	Leu	Leu	His	Arg	Leu	
				55					60	
Lys	Thr	Asn	Asp	Phe	Ala	Pro	Ala	Trp	Ser	
				65					70	
Met	Thr	Arg	Asn	Leu	Phe	Pro	His	Leu	Phe	
				75					80	
Gln	Asn	Pro	Asn	Ser	Thr	Ile	Glu	Ser	Pro	
				85					90	
Leu	Trp	Ala	Leu	Arg	Val	Ile	Leu	Ala	Ala	
				95					100	
Gly	Ile	Gln	Asp	Gln	Leu	Ile	Asp	Gln	Ser	
				105					110	
Leu	Ile	Glu	Pro	Leu	Ala	Gly	Ala	Leu	Gly	
				115					120	
Leu	Ile	Ser	Asp	Trp	Leu	Leu	Thr	Thr	Asn	
				125					130	
Thr	Asn	His	Phe	Asn	Met	Arg	Thr	Gln	Arg	
				135					140	
Val	Lys	Glu	Gln	Leu	Ser	Leu	Lys	Met	Leu	
				145					150	
Ser	Leu	Ile	Arg	Ser	Asn	Ile	Leu	Lys	Phe	
				155					160	
Ile	Asn	Lys	Leu	Asp	Ala	Leu	His	Val	Val	
				165					170	
Asn	Tyr	Asn	Gly	Leu	Leu	Ser	Ser	Ile	Glu	
				175					180	
Ile	Gly	Thr	Gln	Asn	His	Thr	Ile	Ile	Ile	
				185					190	
Thr	Arg	Thr	Asn	Met	Gly	Phe	Leu	Val	Glu	
				195					200	
Leu	Gln	Glu	Pro	Asp	Lys	Ser	Ala	Met	Asn	
				205					210	
Arg	Met	Lys	Pro	Gly	Pro	Ala	Lys	Phe	Ser	
				215					220	
Leu	Leu	His	Glu	Ser	Thr	Leu	Lys	Ala	Phe	
				225					230	
Thr	Gln	Gly	Ser	Ser	Thr	Arg	Met	Gln	Ser	
				235					240	
Leu	Ile	Leu	Glu	Phe	Asn	Ser	Ser	Leu	Ala	
				245					250	
Ile										

<210> 20

<211> 324

<212> PRT

<213> Ebola Zaire

<220>

<400> 20

Met	Glu	Ala	Ser	Tyr	Glu	Arg	Gly	Arg	Pro	1	5	10
Arg	Ala	Ala	Arg	Gln	His	Ser	Arg	Asp	Gly	15	20	25
His	Asp	His	His	Val	Arg	Ala	Arg	Ser	Ser	30	35	40
Ser	Arg	Glu	Asn	Tyr	Arg	Gly	Glu	Tyr	Arg	45	50	55
Gln	Ser	Arg	Ser	Ala	Ser	Gln	Val	Arg	Val	60	65	70
Pro	Thr	Val	Phe	His	Lys	Lys	Arg	Val	Glu	75	80	85
Pro	Leu	Thr	Val	Pro	Pro	Ala	Pro	Lys	Asp	90	95	100
Ile	Cys	Pro	Thr	Leu	Lys	Lys	Gly	Phe	Leu	105	110	115
Cys	Asp	Ser	Ser	Phe	Cys	Lys	Lys	Asp	His	120	125	130
Gln	Leu	Glu	Ser	Leu	Thr	Asp	Arg	Glu	Leu	135	140	145
Leu	Leu	Leu	Ile	Ala	Arg	Lys	Thr	Cys	Gly	150	155	160
Ser	Val	Glu	Gln	Gln	Leu	Asn	Ile	Thr	Ala	165	170	175
Pro	Lys	Asp	Ser	Arg	Leu	Ala	Asn	Pro	Thr	180	185	190
Ala	Asp	Asp	Phe	Gln	Gln	Glu	Glu	Gly	Pro	195	200	205
Lys	Ile	Thr	Leu	Leu	Thr	Leu	Ile	Lys	Thr	210	215	220
Ala	Glu	His	Trp	Ala	Arg	Gln	Asp	Ile	Arg	225	230	235
Thr	Ile	Glu	Asp	Ser	Lys	Leu	Arg	Ala	Leu	240	245	250
Leu	Thr	Leu	Cys	Ala	Val	Met	Thr	Arg	Lys	255	260	265
Phe	Ser	Lys	Ser	Gln	Leu	Ser	Leu	Leu	Cys	270	275	280
Glu	Thr	His	Leu	Arg	Arg	Glu	Gly	Leu	Gly	285	290	295
Gln	Asp	Gln	Ala	Glu	Pro	Val	Leu	Glu	Val	300	305	310
Tyr	Gln	Arg	Leu	His	Ser	Asp	Lys	Gly	Gly	315	320	325
Ser	Phe	Glu	Ala	Ala	Leu	Trp	Gln	Gln	Trp	330	335	340
Asp	Leu	Gln	Ser	Leu	Ile	Met	Phe	Ile	Thr	345	350	355

Ala	Phe	Leu	Asn	Ile	Ala	Leu	Gln	Leu	Pro	
				245						250
Cys	Glu	Ser	Ser	Ala	Val	Val	Val	Ser	Gly	
				255						260
Leu	Arg	Thr	Leu	Val	Pro	Gln	Ser	Asp	Asn	
				265						270
Glu	Glu	Ala	Ser	Thr	Asn	Pro	Gly	Thr	Cys	
				275						280
Ser	Trp	Ser	Asp	Glu	Gly	Thr	Ser	Ile	Gln	
				285						290
Gln	Gln	Leu	Ala	Ser	Cys	Leu	His	Arg	Thr	
				295						300
Arg	Gly	Asp	Trp	His	Ala	Ala	Leu	Lys	Phe	
				305						310
Leu	Phe	Tyr	Phe	Ser	Phe	Leu	Phe	Arg	Ile	
				315						320
Gly	Phe	Cys	Phe							

<210> 21

<211> 340

<212> PRT

<213> Ebola Zaire

<220>

<400> 21

Met	Thr	Thr	Arg	Thr	Lys	Gly	Arg	Gly	His	
1				5					10	
Thr	Ala	Ala	Thr	Thr	Gln	Asn	Asp	Arg	Met	
				15					20	
Pro	Gly	Pro	Glu	Leu	Ser	Gly	Trp	Ile	Ser	
				25					30	
Glu	Gln	Leu	Met	Thr	Gly	Arg	Ile	Pro	Val	
				35					40	
Ser	Asp	Ile	Phe	Cys	Asp	Ile	Glu	Asn	Asn	
				45					50	
Pro	Gly	Leu	Cys	Tyr	Ala	Ser	Gln	Met	Gln	
				55					60	
Gln	Thr	Lys	Pro	Asn	Pro	Lys	Thr	Arg	Asn	
				65					70	
Ser	Gln	Thr	Gln	Thr	Asp	Pro	Ile	Cys	Asn	
				75					80	
His	Ser	Phe	Glu	Glu	Val	Val	Gln	Thr	Leu	
				85					90	
Ala	Ser	Leu	Ala	Thr	Val	Val	Gln	Gln	Gln	
				95					100	
Thr	Ile	Ala	Ser	Glu	Ser	Leu	Glu	Gln	Arg	
				105					110	
Ile	Thr	Ser	Leu	Glu	Asn	Gly	Leu	Lys	Pro	

	115		120
Val Tyr Asp Met	Ala Lys Thr Ile Ser	Ser	
	125		130
Leu Asn Arg Val	Cys Ala Glu Met Val	Ala	
	135		140
Lys Tyr Asp Leu	Leu Val Met Thr Thr	Gly	
	145		150
Arg Ala Thr Ala	Thr Ala Ala Ala Thr	Glu	
	155		160
Ala Tyr Trp Ala	Glu His Gly Gln Pro	Pro	
	165		170
Pro Gly Pro Ser	Leu Tyr Glu Glu Ser	Ala	
	175		180
Ile Arg Gly Lys	Ile Glu Ser Arg Asp	Glu	
	185		190
Thr Val Pro Gln	Ser Val Arg Glu Ala	Phe	
	195		200
Asn Asn Leu Asn	Ser Thr Thr Ser Leu	Thr	
	205		210
Glu Glu Asn Phe	Gly Lys Pro Asp Ile	Ser	
	215		220
Ala Lys Asp Leu	Arg Asn Ile Met Tyr	Asp	
	225		230
His Leu Pro Gly	Phe Gly Thr Ala Phe	His	
	235		240
Gln Leu Val Gln	Val Ile Cys Lys Leu	Gly	
	245		250
Lys Asp Ser Asn	Ser Leu Asp Ile Ile	His	
	255		260
Ala Glu Phe Gln	Ala Ser Leu Ala Glu	Gly	
	265		270
Asp Ser Pro Gln	Cys Ala Leu Ile Gln	Ile	
	275		280
Thr Lys Arg Val	Pro Ile Phe Gln Asp	Ala	
	285		290
Ala Pro Pro Val	Ile His Ile Arg Ser	Arg	
	295		300
Gly Asp Ile Pro	Arg Ala Cys Gln Lys	Ser	
	305		310
Leu Arg Pro Val	Pro Pro Ser Pro Lys	Ile	
	315		320
Asp Arg Gly Trp	Val Cys Val Phe Gln	Leu	
	325		330
Gln Asp Gly Lys	Thr Leu Gly Leu Lys	Ile	
	335		340

<210> 22

<211> 326

<212> PRT

<213> Ebola Zaire

<220>

<400> 22

Met	Arg	Arg	Val	Ile	Leu	Pro	Thr	Ala	Pro	
1				5					10	
Pro	Glu	Tyr	Met	Glu	Ala	Ile	Tyr	Pro	Val	
				15					20	
Arg	Ser	Asn	Ser	Thr	Ile	Ala	Arg	Gly	Gly	
				25					30	
Asn	Ser	Asn	Thr	Gly	Phe	Leu	Thr	Pro	Glu	
				35					40	
Ser	Val	Asn	Gly	Asp	Thr	Pro	Ser	Asn	Pro	
				45					50	
Leu	Arg	Pro	Ile	Ala	Asp	Asp	Thr	Ile	Asp	
				55					60	
His	Ala	Ser	His	Thr	Pro	Gly	Ser	Val	Ser	
				65					70	
Ser	Ala	Phe	Ile	Leu	Glu	Ala	Met	Val	Asn	
				75					80	
Val	Ile	Ser	Gly	Pro	Lys	Val	Leu	Met	Lys	
				85					90	
Gln	Ile	Pro	Ile	Trp	Leu	Pro	Leu	Gly	Val	
				95					100	
Ala	Asp	Gln	Lys	Thr	Tyr	Ser	Phe	Asp	Ser	
				105					110	
Thr	Thr	Ala	Ala	Ile	Met	Leu	Ala	Ser	Tyr	
				115					120	
Thr	Ile	Thr	His	Phe	Gly	Lys	Ala	Thr	Asn	
				125					130	
Pro	Leu	Val	Arg	Val	Asn	Arg	Leu	Gly	Pro	
				135					140	
Gly	Ile	Pro	Asp	His	Pro	Leu	Arg	Leu	Leu	
				145					150	
Arg	Ile	Gly	Asn	Gln	Ala	Phe	Leu	Gln	Glu	
				155					160	
Phe	Val	Leu	Pro	Pro	Val	Gln	Leu	Pro	Gln	
				165					170	
Tyr	Phe	Thr	Phe	Asp	Leu	Thr	Ala	Leu	Lys	
				175					180	
Leu	Ile	Thr	Gln	Pro	Leu	Pro	Ala	Ala	Thr	
				185					190	
Trp	Thr	Asp	Asp	Thr	Pro	Thr	Gly	Ser	Asn	
				195					200	
Gly	Ala	Leu	Arg	Pro	Gly	Ile	Ser	Phe	His	
				205					210	
Pro	Lys	Leu	Arg	Pro	Ile	Leu	Leu	Pro	Asn	
				215					220	
Lys	Ser	Gly	Lys	Lys	Gly	Asn	Ser	Ala	Asp	
				225					230	
Leu	Thr	Ser	Pro	Glu	Lys	Ile	Gln	Ala	Ile	
				235					240	
Met	Thr	Ser	Leu	Gln	Asp	Phe	Lys	Ile	Val	
				245					250	

Pro	Ile	Asp	Pro	Thr	Lys	Asn	Ile	Met	Gly	
				255						260
Ile	Glu	Val	Pro	Glu	Thr	Leu	Val	His	Lys	
				265						270
Leu	Thr	Gly	Lys	Lys	Val	Thr	Ser	Lys	Asn	
				275						280
Gly	Gln	Pro	Ile	Ile	Pro	Val	Leu	Leu	Pro	
				285						290
Lys	Tyr	Ile	Gly	Leu	Asp	Pro	Val	Ala	Pro	
				295						300
Gly	Asp	Leu	Thr	Met	Val	Ile	Thr	Gln	Asp	
				305						310
Cys	Asp	Thr	Cys	His	Ser	Pro	Ala	Ser	Leu	
				315						320
Pro	Ala	Val	Ile	Glu	Lys					
				325						

<210> 23

<211> 288

<212> PRT

<213> Ebola Zaire

<220>

<400> 23

Met	Glu	Ala	Ser	Tyr	Glu	Arg	Gly	Arg	Pro	
1				5					10	
Arg	Ala	Ala	Arg	Gln	His	Ser	Arg	Asp	Gly	
				15					20	
His	Asp	His	His	Val	Arg	Ala	Arg	Ser	Ser	
				25					30	
Ser	Arg	Glu	Asn	Tyr	Arg	Gly	Glu	Tyr	Arg	
				35					40	
Gln	Ser	Arg	Ser	Ala	Ser	Gln	Val	Arg	Val	
				45					50	
Pro	Thr	Val	Phe	His	Lys	Lys	Arg	Val	Glu	
				55					60	
Pro	Leu	Thr	Val	Pro	Pro	Ala	Pro	Lys	Asp	
				65					70	
Ile	Cys	Pro	Thr	Leu	Lys	Lys	Gly	Phe	Leu	
				75					80	
Cys	Asp	Ser	Ser	Phe	Cys	Lys	Lys	Asp	His	
				85					90	
Gln	Leu	Glu	Ser	Leu	Thr	Asp	Arg	Glu	Leu	
				95					100	
Leu	Leu	Leu	Ile	Ala	Arg	Lys	Thr	Cys	Gly	
				105					110	
Ser	Val	Glu	Gln	Gln	Leu	Asn	Ile	Thr	Ala	
				115					120	
Pro	Lys	Asp	Ser	Arg	Leu	Ala	Asn	Pro	Thr	
				125					130	

Ala	Asp	Asp	Phe	Gln	Gln	Glu	Glu	Gly	Pro	
				135					140	
Lys	Ile	Thr	Leu	Leu	Thr	Leu	Ile	Lys	Thr	
				145					150	
Ala	Glu	His	Trp	Ala	Arg	Gln	Asp	Ile	Arg	
				155					160	
Thr	Ile	Glu	Asp	Ser	Lys	Leu	Arg	Ala	Leu	
				165					170	
Leu	Thr	Leu	Cys	Ala	Val	Met	Thr	Arg	Lys	
				175					180	
Phe	Ser	Lys	Ser	Gln	Leu	Ser	Leu	Leu	Cys	
				185					190	
Glu	Thr	His	Leu	Arg	Arg	Glu	Gly	Leu	Gly	
				195					200	
Gln	Asp	Gln	Ala	Glu	Pro	Val	Leu	Glu	Val	
				205					210	
Tyr	Gln	Arg	Leu	His	Ser	Asp	Lys	Gly	Gly	
				215					220	
Ser	Phe	Glu	Ala	Ala	Leu	Trp	Gln	Gln	Trp	
				225					230	
Asp	Arg	Gln	Ser	Leu	Ile	Met	Phe	Ile	Thr	
				235					240	
Ala	Phe	Leu	Asn	Ile	Ala	Leu	Gln	Leu	Pro	
				245					250	
Cys	Glu	Ser	Ser	Ala	Val	Val	Val	Ser	Gly	
				255					260	
Leu	Arg	Thr	Leu	Val	Pro	Gln	Ser	Asp	Asn	
				265					270	
Glu	Glu	Ala	Ser	Thr	Asn	Pro	Gly	Thr	Cys	
				275					280	
Ser	Trp	Ser	Asp	Glu	Gly	Thr	Pro			
				285						

<210> 24

<211> 11

<212> PRT

<213> Ebola Zaire

<220>

<400> 24

Val	Tyr	Gln	Val	Asn	Asn	Leu	Glu	Glu	Ile	
1				5					10	
Cys										

<210> 25

<211> 23

<212> PRT

<213> Ebola Zaire

<220>

<400> 25

Leu	Lys	Phe	Ile	Asn	Lys	Leu	Asp	Ala	Leu
1				5					10
Leu	Val	Val	Asn	Tyr	Asn	Gly	Leu	Leu	Ser
				15					20
Ser	Ile	Phe							

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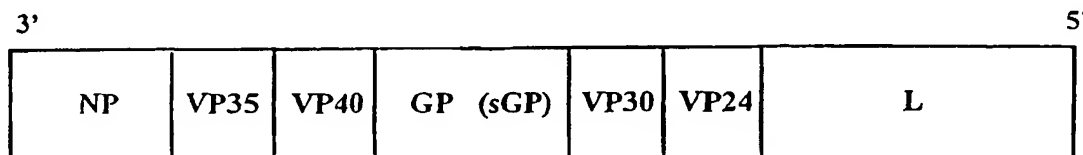
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[Continued on next page]

(54) Title: EBOLA VIRION PROTEINS EXPRESSED FROM VENEZUELAN EQUINE ENCEPHALITIS (VEE) VIRUS REPLI-
CONS

Organization of the Ebola Virus Genome



NP Major Nucleocapsid Protein
VP35 Phosphoprotein
VP40 Membrane-Associated Matrix Protein
GP Transmembrane Glycoprotein
sGP Secreted Glycoprotein
VP30 Ribonucleoprotein Associated (Minor)
VP24 Membrane-Associated Protein (Minor)
L RNA-Dependent RNA Polymerase

(57) Abstract: Using the Ebola GP, NP, VP24, VP30, VP35 and VP40 virion proteins, a method and composition for use in inducing
an immune response which is protective against infection with Ebola virus is described



(15) Information about Correction:

Previous Correction:

see PCT Gazette No. 31/2000 of 3 August 2000, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/14311

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/40 C12N7/01 C07K14/08 C07K16/10 C12Q1/68
G01N33/569 G01N33/577 A61K39/12 A61K31/7105

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L. XU ET AL.: "Immunization for Ebola virus infection" NATURE MEDICINE, vol. 4, no. 1, January 1998 (1998-01), pages 37-42, XP002131515 NATURE PUBLISHING CO., NY, US the whole document ---	1,7, 13-22, 25, 36-46,52
X	L. VANDERZANDEN ET AL.: "DNA vaccines expressing either the GP or NP genes of Ebola virus protect mice from lethal challenge" VIROLOGY, vol. 246, no. 1, 20 June 1998 (1998-06-20), pages 134-144, XP002131516 ACADEMIC PRESS, INC., NEW YORK, US the whole document ---	1,7, 13-22, 25, 36-46,52

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *&* document member of the same patent family

Date of the actual completion of the international search

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Name and mailing address of the ISA

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NL - 2280 HV Rijswijk

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/14311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US PUSHKO, PETER (1) ET AL: "Venezuelan equine encephalitis virus replicon vector: Immunogenicity studies with ebola NP and GP genes in guinea pigs." retrieved from STN Database accession no. V199799764569 XP002131517 abstract & BROWN, F. [EDITOR]; BURTON, D. [EDITOR]; DOHERTY, P. [EDITOR]; MEKALANOS, J. [EDITOR]. VACCINES (COLD SPRING HARBOR), (1997) VOL. 97, PP. 253-258. VACCINES (COLD SPRING HARBOR); MOLECULAR APPROACHES TO THE CONTROL OF INFECTIOUS DISEASES. PUBLISHER: CO,</p>	<p>1,7, 13-25, 31,33, 34,56</p>
X	<p>WO 96 37616 A (UNIV NORTH CAROLINA ;US HEALTH (US); JOHNSTON ROBERT E (US); DAVIS) 28 November 1996 (1996-11-28)</p> <p>page 8, line 29 -page 9, line 2; claims 1-37</p>	<p>1,7, 13-25, 31,33, 34,56</p>
X	<p>SANCHEZ A ET AL: "SEQUENCE ANALYSIS OF THE EBOLA VIRUS GENOME: ORGANIZATION, GENETIC ELEMENTS, AND COMPARISON WITH THE GENOME OF MARBURG VIRUS" VIRUS RESEARCH,NL,AMSTERDAM, vol. 29, no. 3, page 215-240 XP000198438 ISSN: 0168-1702</p>	<p>1,7, 13-22,25</p>
Y	<p>figure 8</p>	<p>31,33, 34, 36-46, 52,56</p>
X	<p>A. SANCHEZ ET AL.: "Variation in the glycoprotein and VP35 genes of Marburg virus strains" VIROLOGY, vol. 240, no. 1, 5 January 1998 (1998-01-05), pages 138-146, XP002128208 ACADEMIC PRESS, INC.,NEW YORK, US</p>	<p>1,7, 13-22,25</p>
Y	<p>the whole document</p>	<p>31,33, 34, 36-46, 52,56</p>

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/14311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>P. PUSHKO ET AL.: "Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: Expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo" VIROLOGY, vol. 239, 1997, pages 389-401, XP002128209 ACADEMIC PRESS, INC., NEW YORK, US the whole document</p> <p style="text-align: center;">---</p>	<p>31,33, 34, 36-46, 52,56</p>
E	<p>WO 99 32147 A (UNIV MICHIGAN ;NABEL GARY J (US); SANCHEZ ANTHONY (US)) 1 July 1999 (1999-07-01) the whole document</p> <p style="text-align: center;">-----</p>	<p>1,7, 13-22,25</p>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/14311

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,7,31,46,54,55 (complete); 13-25,33-45,52,53,56-59 (partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: (1,7,31,46,54,55)-complete; (13-25,33-45,52,53,56-59)-partially

A DNA fragment which encodes a GP Ebola protein, said DNA fragment comprising the sequence specified in SEQ ID No.1; a recombinant DNA construct comprising (i) a vector and (ii) said DNA; said recombinant DNA construct wherein said vector is a VEE virus replication vector; said Ebola virus DNA fragments are from Ebola Zaire 1976; a host cell comprising said recombinant DNA construct; said construct wherein said construct is for VRepEboGP; infectious alphavirus particles produced from packaging said self replicating RNA; a pharmaceutical composition comprising said alphavirus particle; a vaccine for Ebola comprising said alphavirus particle; a method for producing Ebola virus protein GP; an isolated and purified Ebola GP protein specified in SEQ ID No.17; an antibody to a peptide encoded by said SEQ ID No.17; a method for detecting Ebola virus infection;

2. Claims: (2,8,30,47)-complete; (13-25,33-45,52,53,56-60)-partially

Idem as invention 1 but limited to NP respectively SEQ ID Nos. 2 and 18;

3. Claims: (3,9,26,48)-complete; (13-25,33-45,52,53,56-60)-partially

Idem as invention 1 but limited to VP24 respectively SEQ ID Nos. 3 and 19;

4. Claims: (4,10,27,32,49)-complete; (13-25,33-45,52,53,56-59)-partially

Idem as invention 1 but limited to VP30 respectively SEQ ID Nos. 4,7,20 and 23;

5. Claims: (5,11,28,50)-complete; (13-25,33-45,52,53,56-59)-partially

Idem as invention 1 but limited to VP35 respectively SEQ ID Nos. 5 and 21;

6. Claims: (6,12,29,51)-complete; (13-25,33-45,52,53,

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Idem as invention 1 but limited to VP40 respectively SEQ ID
Nos. 6 and 22;

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 99/14311

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9637616 A	28-11-1996	US 5792462 A	11-08-1998
		AU 5925696 A	11-12-1996
		CA 2220964 A	28-11-1996
		JP 11505719 T	25-05-1999

WO 9932147 A	01-07-1999	NONE	
